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<p>13. ABSTRACT (Maximum 200)</p> <p>The experiments described here identified components of the pathways through which Myc acts to both increase proliferation and induce apoptosis in mammary epithelial cells (MECs). The accelerated growth of MECs which overexpress c-myc was due to a more rapid passage through the G₁ phase of the cell cycle. A1N4-myc cells displayed constitutive hyperphosphorylation of the retinoblastoma protein, most likely as a result of elevated cdk2 activity.</p> <p>The high propensity of Myc83 cells to undergo apoptosis was associated with constitutive expression of Bax and p53. In contrast, Bcl-X_L expression varied significantly with growth factor addition or removal. Bcl-X_L protein levels were dramatically elevated in cells which were grown in the presence of EGF or TGFα compared to apoptotic cells which had been deprived of EGF or treated with TGFβ. Mammary tumors arising in Myc/TGFα double transgenic mice also consistently showed a higher relative ratio of Bcl-X_L/Bax than tumors from Myc single transgenic mice. Our results suggest an important role of Bcl-X_L in the ability of EGF to act as a survival factor for MECs which overexpress c-Myc</p>					
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Robert B. Dr. 9/26/96
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INTRODUCTION

The proto-oncogene *c-myc* is frequently amplified in many human cancers including breast cancer, and its amplification is associated with a high proliferation rate and poor prognosis (1-8). Transgenic animal models have confirmed the tumorigenic potential of *c-myc* overexpression in mammary tissue (9-13), but the mechanism by which *Myc* promotes tumor growth has not been elucidated. De-regulated *c-myc* expression can promote cell transformation in cooperation with growth factors such as EGF (14-16), and has been associated with cell proliferation as well as apoptosis (17-21). Thus, the experiments described here were intended to identify components of the pathways through which *Myc* acts to both increase proliferation and induce apoptosis in mammary epithelial cells and to examine the impact of EGF on those pathways. The studies have focused primarily on two mammary epithelial cell lines (MECs): 1). *Myc*#83 cells (22) were derived from a mammary tumor of a *Myc* transgenic mouse and can be induced to undergo apoptosis by altering their growth environment (i.e. removal of the growth/survival factor EGF or addition of the growth inhibitor TGF β). 2). A1N4-*myc* cells (23) are chemically immortalized human MECs which demonstrate an accelerated growth rate compared to parental cells but undergo reversible growth arrest rather than apoptosis in the absence of EGF.

By using these two types of cell lines, both aspects of *Myc* overexpression have been examined in MECs.

BODY

Materials and Methods

Cell lines

The benzo(a)pyrene-immortalized and transformed human mammary epithelial cell line 184A1N4-*myc* (14, 23) and its parental cell line 184A1N4 were used to study the effects of *myc* overexpression on cell cycle regulation. The A1N4-*myc* line was established via retroviral infection with a construct containing mouse *myc* under the control of the Moloney mouse

leukemia virus long terminal repeat (MMLV LTR). Both cell lines were maintained in media containing 0.5% fetal calf serum (FCS), 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, and 10 ng/ml epidermal growth factor (EGF, Upstate Biotechnology Incorporated, Lake Placid NY). The cells arrest in G1 in the absence of EGF.

The Myc#83 cell line was established from a mammary tumor of a transgenic mouse (MMTV-c-myc M) (22). The TGF α /Myc#75 line was derived from a tumor arising in a double transgenic virgin female. In those cell lines, myc expression is driven by the mouse mammary tumor virus promoter. The cells were routinely grown in media containing 2.5% FCS, 10 ng/ml /EGF and 5 mg/ml insulin. Myc83 cells undergo apoptosis when EGF is removed from their culture media.

Cell Death ELISA

Cells were plated in 6-well plates (50,000 cells/well) in normal growth media. The next day, TGF β was added to the media or the cells were switched to media without EGF. Twenty-four hours later, the cells were harvested by trypsinization and centrifugation. The cell pellets were resuspended in lysis buffer and kept on ice for 30 minutes. The nuclei were removed by a 10 minute centrifugation and cytoplasmic lysates were stored at -70 °C.

Cytoplasmic apoptotic DNA fragments were detected using an ELISA kit (Boehringer Mannheim) with antibodies directed against histones and DNA. The 96-well plate was coated overnight (4 °C) with the first antibody (anti-histone) and then incubated with lysis buffer for 30 minutes at room temperature (RT). The wells were washed 3 times and then incubated with 100 µl cytoplasmic lysate for 90 minutes (RT). The wells were washed again 3 times and then incubated with the peroxidase-linked second antibody (anti-DNA). Following the final wash, ABTS peroxidase substrate was added. After a 10 minute incubation, color development was detected by measuring absorbance at 410 nm.

Morphological Assessment of Apoptosis

Cells were plated onto sterile glass cover slips in 6 well plates (1.7×10^5 cells/well) and treated as above. After 24 h, cells were rinsed with PBS and fixed for 10 minutes in PBS containing 10% formalin and 0.1% Triton-x 100. Cells were then rinsed with PBS, stained for 10 min with Hoechst dye #33258 (10 μ g/ml, Polysciences, Inc., Warrington, PA), and washed three times with PBS.

Northern analysis

Cells were plated in 60 mm plates (5×10^5 cells/ plate) and treated as above for 24 h. Total RNA was harvested by the guanidine thiocyanate-acid phenol method (24) and pellets were dissolved in 40 μ l diethyl pyrocarbonate-treated water. Total RNA (15 μ g) was separated on 1% agarose gels containing formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL) and fixed by UV irradiation. Blots were sequentially hybridized overnight with the following 32 P-labeled, random-primed murine probes: bcl-2 (nt 1635-1945), bcl-x_{L+S} (mRNA nt 110-394), bax (mRNA nt 138-389), and p53 (nt 97-1407).

RT-PCR

The relative amounts of bcl-x_L and bcl-x_S mRNA were measured by an RT-PCR assay. One μ g of RNA from each sample was reverse transcribed with random primers and the cDNA for bcl-x_{L+S} was amplified for 31 cycles using a pair of primers that amplify the nucleotide sequence containing the region differentially spliced in the bcl-x_L and bcl-x_S mRNAs. The 5' primer corresponded to bcl-x mRNA nt 466-488 (5' -GCG CGG GAG GTG ATT CCC ATG GC-3') and the 3' primer corresponded to bcl-x mRNA nt 891-870 (5' -CAT GCC CGT CAG GAA CCA GCG G-3'). The PCR products were fractionated on a 2% agarose gel, transferred to a nylon membrane, and fixed by UV irradiation. Expression of bcl-x_L was identified by hybridizing the membrane overnight with a 32 P-labelled random-primed probe for bcl-x_L (bcl-x mRNA nt 466-891). Expression of bcl-x_S was identified by hybridizing the membrane overnight with an oligonucleotide specific for the splice site contained within the 237-bp bcl-x_S product (5' -CAG AGC TTT GAG CAG GAC ACT TTT

GTG G-3'). Autoradiograph exposure times were: bcl-x_L, 3 h; bcl-x_S, 20 h. The signal obtained was proportional to the RNA input and the number of PCR cycles.

Western Analysis

Myc83 cells were plated in 100 mm plates (1.3×10^6 cells/plate) and treated as above for 24 h. Cells were then rinsed with cold phosphate-buffered saline (PBS) and lysed in RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). Lysates were also prepared from mouse mammary tumors. Frozen tumors were pulverized in liquid nitrogen and homogenized in Tris-SDS (10 mM, pH 7.4, 1%). Lysates were then boiled for 5 min, diluted 1 to 1 with 2x loading buffer, and frozen at -70 °C. Twenty µg of protein were separated on 14% SDS-PAGE gels (or 10% for examination of p53) and transferred to nitrocellulose. Blots were blocked with 5% milk in Tris-buffered saline with Tween-20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween-20) for 1 h at room temperature and then incubated in TBST with 1% BSA and the following antibodies (diluted 1/400): Bcl-x_{L+S} (S-18), Bax (N-20), and Bcl-2 (N-19) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and p53 (Ab-1, Oncogene Sciences, Cambridge, MA). Proteins were visualized with an HRP-linked second antibody (1/500 in TBST with 1% BSA) and a chemiluminescent detection system (Pierce, Rockford, IL). In some cases, antibodies were removed from the blots by incubation in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, 2 % SDS) for 30 min (50 °C) and the blots were re-probed. Amido black staining of the membranes demonstrated equal loading and transfer of the samples.

A1N4 and A1N4-myc cells were plated, arrested, and restimulated with EGF as previously described. At 1.5 or 3 hour intervals following EGF stimulation, total cell lysates were prepared. Cells were washed with cold PBS and then scraped into cold lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton x-100, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM Na₃O₄V, 100 mM NaF, 10 mM pyrophosphate, 10 µg/ml PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). After a 10 minute incubation on ice, lysates were spun for 10 minutes in a cold micocentrifuge to remove cellular debris and were frozen at -70 °C. Twenty µg of protein

from each sample were separated by SDS-PAGE and transferred to either nitrocellulose or PVDF membranes. Acrylamide concentrations varied depending on the target protein as follows; Rb, 6%; cyclin D1, cyclin E, cdk2, and cdk4, 10%; p21 and p27, 14%. Blots were blocked in 4% milk, 1% BSA in TBST for 1 h at room temperature and then incubated in TBST with 1% BSA and the following antibodies (1 µg/ml): Rb (Pharmingen), cyclin D1 and E (UBI), cdk2 and cdk4 (Santa Cruz), or p27 (Santa Cruz). Proteins were visualized with an HRP-linked second antibody (1/2000 in TBST with 1% BSA) and a chemiluminescent detection system. Amido black or India ink staining of the membranes demonstrated equal loading and transfer of the samples.

Kinase assays

Cell lysates (100 µg) were incubated with 1 µg anti-cdk2 antibody for 2 h (4°C) prior to precipitation with Agarose A beads (Santa Cruz). Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (50 mM HEPES [pH 7.5], 10 mM MgCl₂, 1 mM EDTA, 1 mM NaF, 1 mM DTT, 0.3 mM β-glycerophosphate, 1 mM Na₃O₄ V, 10 µg/ml PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin). The beads were then resuspended in 30 µl kinase buffer and the reaction was started by adding ATP (200 µM), γ³²P-ATP (5 µCi) and histone H1 (1 µg). Samples were incubated at 30 °C for 15 min before stopping the reaction with 2x loading buffer (62.5 mM Tris [pH 6.8], 10% sucrose, 2% SDS, 5% β-mercaptoethanol, 1% bromphenol blue). Labeled proteins were run on a 10% polyacrylamide gel which was dried prior to visualization with a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale CA).

Inhibition of EGFR tyrosine kinase activity by PD 153035

PD153035 (Park Davis) has been shown to specifically down regulate the tyrosine kinase activity of the EGFR (25). The TGFα/Myc#75 cells were treated with PD 153035 (10 µM) for 24 h before harvesting cytoplasmic lysates for apoptosis ELISA or whole cell lysates for Bcl-x_L Western analysis .

Densitometry

Integrated optical densities of bands visualized on film after Northern or Western analysis were obtained with the ScanJet Plus scanner (Hewlett Packard) and the IP Lab Gel image analysis program (Signal Analytics Corporation, Vienna, VA).

Results

Myc and cell cycle regulation

We previously showed that c-myc overexpression in both mouse (HC-14-myc and MMEC-myc) and human (A1N4-myc) MECs decreased the doubling time by about 6 h compared to parental lines. Experiments with the A1N4 lines suggested that the difference was not due to increased sensitivity to EGF, but rather to a shortening of G₁. A1N4 and A1N4-myc cells were arrested in G₁ in the absence of EGF and were allowed to re-enter the cell cycle by replacing EGF. FACS analysis demonstrated that the A1N4-myc cells began to enter S phase 12 h after EGF addition and percent cells in S phase peaked at 18 h. In contrast, parental cells did not enter S phase until 18 h and peaked at 24 h.

The shortened G₁ phase does not appear to be due to any gross changes in cyclin A or D1 RNA expression as assessed by a non-radioactive RNase protection assay which we have developed (26). In unsynchronized cells, *myc* overexpression had no significant effect on cyclin A or D1 mRNA expression. Cyclin mRNA was nearly undetectable in arrested cells and induction was closely correlated with changes in cell cycle phase.

New Results: A significant difference between the two cell lines was observed with respect to Rb expression and phosphorylation. In arrested A1N4 cells, Rb expression was relatively low and the protein was present only in the hypophosphorylated state (Figure 1). About 6 hours after EGF stimulation, approximately 50% of the protein was found in the hyperphosphorylated state. At all time points beyond 6 hours, Rb protein levels were greatly increased and most of the protein was hyperphosphorylated. In contrast, Rb was highly expressed and phosphorylated at all timepoints tested in A1N4-myc cells.

Since Rb plays an important role in the G₁ phase of the cell cycle, we next examined the expression of several proteins known to be involved in the regulation of G₁ progression (Figure 2). In agreement with the RPA results, cyclin D1 protein expression was very low in arrested cells, was rapidly induced following EGF stimulation, and remained elevated throughout the remainder of the cell cycle. Cyclin D1 levels were maximal at 6 h after stimulation in A1N4 cells, and at 3 h in A1N4-myc cells. Cyclin E protein was detectable in arrested cells of both lines, but was further stimulated by EGF addition and then down regulated later in the cell cycle. Peak levels of this cyclin were observed between 9 and 15 h in parental cells, and from 3 to 6 h in c-myc-expressing cells. Expression of two cyclin dependent kinases which interact with cyclins D1 and E were also examined. Cdk4 expression was constant throughout the cell cycle in both cell lines, but protein levels were higher in A1N4-myc cells. Western analysis of cdk2 demonstrated a dramatic mobility shift in the protein due to CAK phosphorylation. In A1N4 cells, the shift was observed about 12 h after EGF addition, while A1N4-myc cells already contained low levels of phosphorylated protein even when arrested, with a maximal shift at about 6-9 h post stimulation. As expected, those mobility shifts corresponded to the time of cyclin E induction. Finally, expression of a cdk inhibitor, p27, was analyzed. Protein levels were quite high in arrested A1N4 cells and decreased as the cells progressed through the cell cycle. In contrast, p27 was barely detectable in arrested A1N4-myc cells, and was rapidly eliminated following EGF addition.

The results presented in Figure 2 suggested that differences in cdk2 activity might be responsible for the shortened G₁ phase in c-myc-overexpressing cells. We therefore directly examined activation of cdk2 in the cells with an *in vitro* kinase assay (Figure 3). As predicted, arrested parental cells contained very little active cdk2, and a major increase in activity was observed 12 hours after EGF stimulation, the time at which cyclin E was maximally expressed, p27 levels were reduced, and cdk2 was phosphorylated by CAK. In contrast, cdk2 was active even in EGF-deprived A1N4-myc cells, with maximal activation at 6 h after EGF stimulation.

Myc and the regulation of apoptosis in MECs

We previously showed Myc83 cells become apoptotic when deprived of EGF or when treated with TGF β in the presence of EGF. This response has been confirmed in additional cell lines derived from mammary tumors of MMTV-myc transgenic mice (not shown). There was no apparent synergism for apoptosis induction between addition of TGF β and removal of EGF. Microscopic examination of cells stained with Hoechst dye also demonstrated that the cells were undergoing morphological changes which are characteristic of apoptotic cells. The time course for TGF β -induced apoptosis was quite similar to that observed when EGF is withdrawn; apoptotic DNA fragments were first detected at about 15 hours after treatment and maximal levels were observed between 24 and 48 hours.

New Results: A 24 h treatment period was chosen to assess changes in gene expression, which was first examined at the RNA level by Northern analysis. RNA blots were sequentially hybridized with murine probes for bcl-2, bcl-x, bax, and p53, as shown in Figure 4. bcl-x showed the most significant changes in expression. Levels were highest in EGF treated cells and were significantly decreased following TGF β treatment (by about 50%) or EGF withdrawal (by about 70%). Overall expression of bcl-2 was much lower than bcl-x, with only very faint bands appearing after a 1 week exposure of the blot to film. However, there was still observable variation in bcl-2 expression, with the strongest signal for cells grown in the presence of EGF. In contrast, bax RNA was easily detectable and expression appeared to be relatively uniform across treatments. Expression of p53 mRNA was also fairly consistent across treatments, with the notable exception of EGF deprivation, which resulted in a 50% reduction in band intensity. RT-PCR was used to distinguish the long and short forms of Bcl-x RNA. The results indicated that the bcl-x signal observed by Northern analysis was largely due to expression of bcl-x_L rather than bcl-x_S, and the treatments did not induce any significant shift in RNA splicing (not shown).

Western blots were used to examine expression at the protein level (Figure 5). Bcl-x_L protein levels varied dramatically, with highest expression in EGF-treated cells, as in the

Northern results. Levels of Bcl-x_L protein were similar in EGF- and TGF α -treated cells. Following TGF β treatment or EGF withdrawal, there was an 80% reduction in Bcl-x_L protein. Analysis of lysates prepared at various time points following growth factor addition or withdrawal indicated that the decrease in Bcl-x_L protein preceded the onset of DNA degradation (not shown). The Bcl-x_S form of the protein could not be detected by Western analysis, reflecting the RT-PCR results described above. Bcl-2 protein was also undetectable by Western analysis, in agreement with the weak bands observed on Northern blots. Bax and p53 protein levels were relatively high and showed very little variation across treatments.

The relative ratios of Bcl-x_L to Bax were also compared in mammary tumors from single (MMTV-myc) or double (MMTV-myc X MT-TGF α) transgenic mice. Relative levels of Bcl-x_L and Bax were measured by Western analysis and relative ratios of the signal intensity for the two proteins were calculated from the values obtained by densitometry (Figure 6). Lysates from Myc single transgenic tumors consistently showed a relative ratio of Bcl-x_L to Bax of approximately 0.5 (0.56 \pm 0.06). Ratios for the double transgenic tumors were more variable, but were consistently higher than the single transgenics, with a mean value of 2.9 (\pm 1.0), a statistically significant difference from Myc tumors ($p < 0.05$). As in the cell lines, Bcl-2 was not detected in the tumor lysates.

Additional evidence for the importance of the TGF α /EGF receptor system in the survival of Myc-overexpressing MECs was provided by using a synthetic inhibitor of EGF receptor tyrosine kinase activity (PD153035). PD153035 has been shown to specifically downregulate the tyrosine phosphorylation status of the EGFR (25). The TGF α /Myc#75 cells became apoptotic when exposed to PD153035 for 24 hours (Figure 7). Removing EGF from the growth media of these cells did not affect viability, but exposure to PD153035 in either the presence or absence of EGF induced apoptosis. In contrast, bFGF (FGF-2), which acts through a different receptor tyrosine kinase, could rescue the cells from the effects of the drug. Western analysis demonstrated that a downstream effect of EGFR inhibition by PD153035 was decreased levels of Bcl-x_L protein, similar to the effect of EGF withdrawal on

Myc#83 cells . Simply removing EGF from the growth media of TGF α /Myc#75 cells did not alter Bcl-x_L levels. The results confirm the observation that these cells are not dependent on exogenous EGF for survival, but their viability is dependent on the EGFR signal transduction pathway.

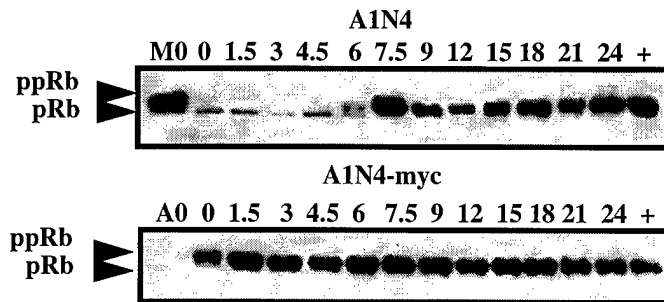


Figure 1: Expression and phosphorylation of Rb in synchronized cells (A1N4 and A1N4-myc). Arrested cells were re-stimulated with EGF and whole cell lysates were prepared at the times indicated. 20 μ g of protein were separated on a 6% gel before transfer to nitrocellulose for Western analysis. The faster moving band is due to hypophosphorylated (inhibitory) Rb and the upper band contains hyperphosphorylated (inactive) Rb. A0=A1N4 at time 0. M0=A1N4-myc at time 0, +=unsynchronized cells.

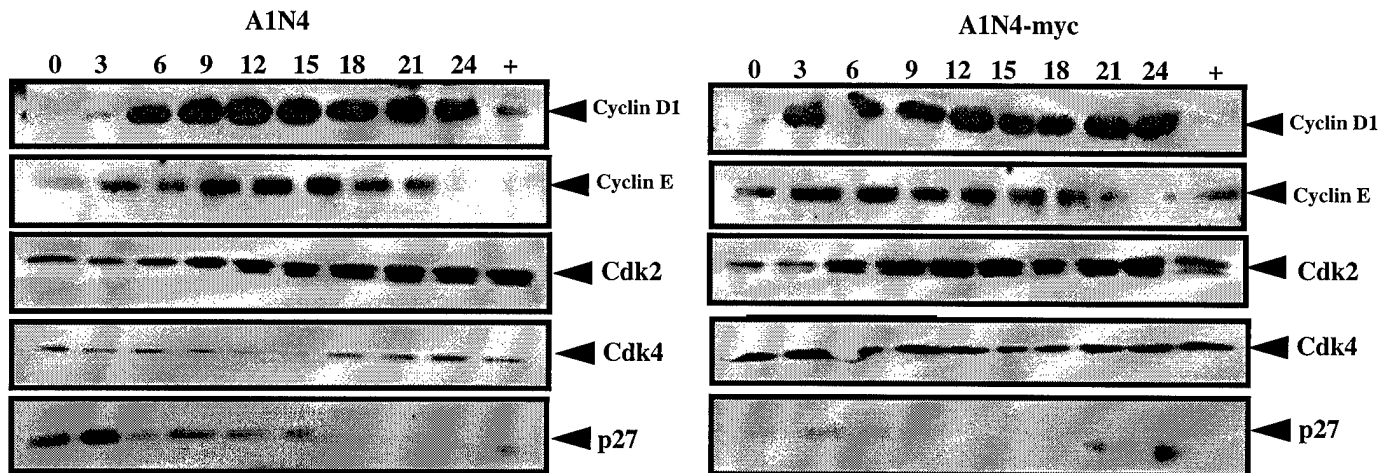


Figure 2: Expression of the G₁ cyclins D1 and E, their associated kinases cdk4 and cdk2, and the cdk inhibitor p27 in synchronized A1N4 and A1N4-myc cells. Lysates were harvested as in Figure 1 and were separated on 10% acrylamide gels prior to transfer to nitrocellulose for western analysis. In the case of cdk2, phosphorylation by CAK leads to a downward shift in mobility, producing the observed doublet. +, unsynchronized cells.

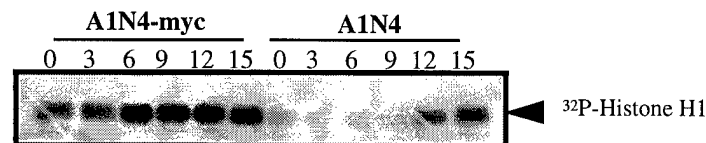


Figure 3: Kinase activity of cdk2 in synchronized A1N4 and A1N4-myc cells. Cdk2 was immunoprecipitated from whole cell lysates at the indicated times following EGF re-stimulation. The precipitates were then incubated for 15 min at 37 °C in the presence of histone H1 and γ ATP. Labeled substrate was detected by phosphorimager analysis following fractionation on a 10% PAGE gel.

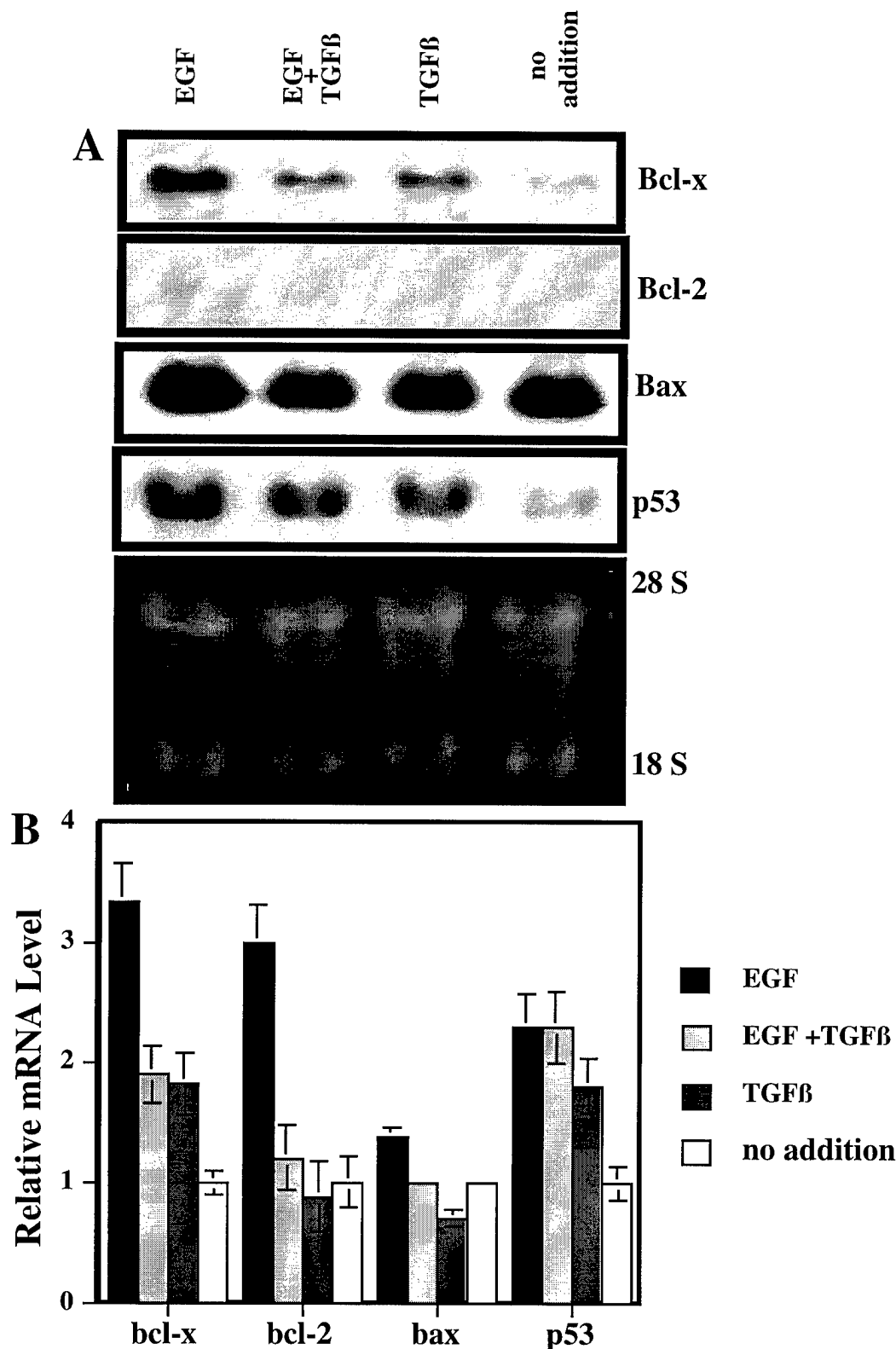


Figure 4: Northern Analysis. Myc#83 cells were treated for 24 h before harvesting total RNA. *Panel A:* Representative results. Northern blots were sequentially hybridized with random-primed probes for mouse bcl-2, bcl-x, bax, and p53. Ethidium bromide-stained 28S and 18S ribosomal RNA bands are shown as a loading standard. Autoradiography exposure times were as follows: bcl-2, 7 days; bcl-x_{L+S}, 5 days; bax, 3 days; p53, 2 days. *Panel B:* Cumulative data. Relative band intensities were determined as described in Materials and Methods. For each gene of interest, the mean band intensity for untreated cells (-) was assigned a value of 1 and all other values were calculated as a relative increase or decrease. n=8 (+/-SE).

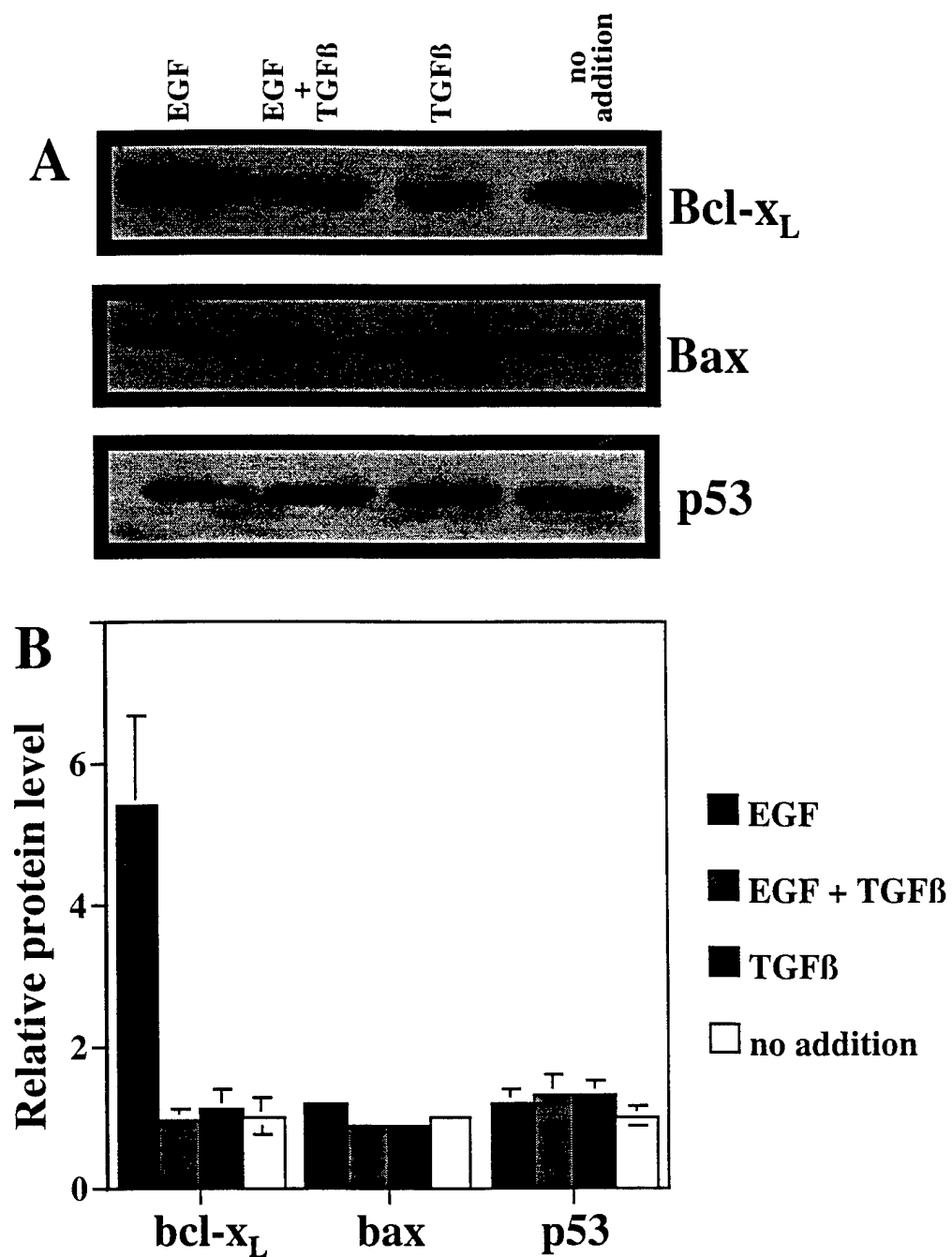


Figure 5:

Western analysis of Myc#83 cells. Cells were treated for 24 h as in Fig 1A before preparing total cell lysates. 20 μ g of protein were separated by SDS-PAGE and transferred to nitrocellulose. **A:** Representative results. Individual blots were incubated with antibodies against Bcl-x, Bax, and p53. **B:** Cumulative data. The mean band intensity for untreated cells was assigned a value of 1 and all other values were calculated as a relative increase or decrease. n=6 (+/-SE).

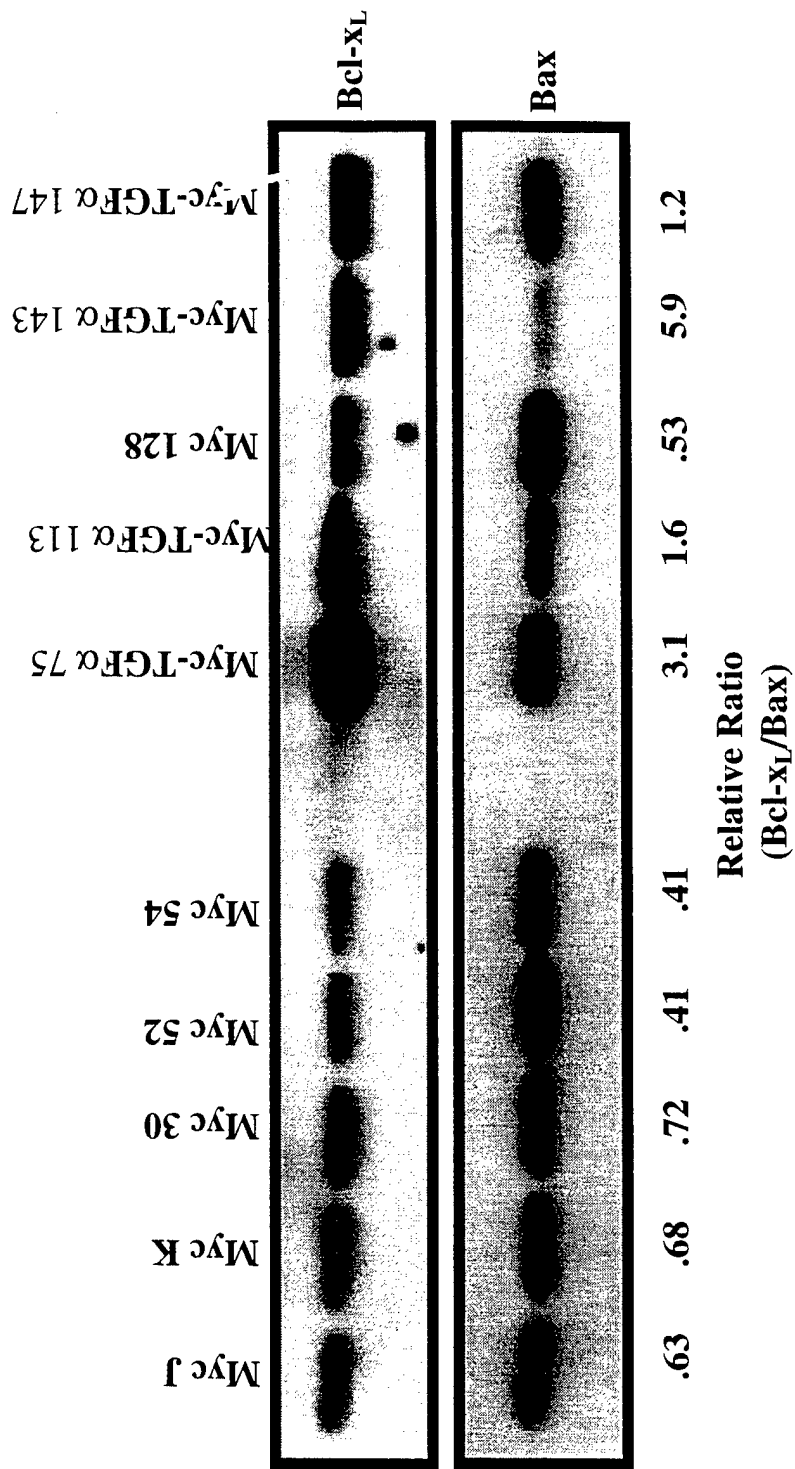


Figure 6: Western analysis of mammary tumors from single Myc and double Myc/TGFα transgenic mice. Lysates were prepared from frozen tumors and 20 µg of protein were separated on 14% polyacrylamide gels. The blot was probed first with an antibody against Bcl-x and then stripped and re-probed with Bax antibody. Relative ratios were calculated from the band intensities as measured by densitometry.

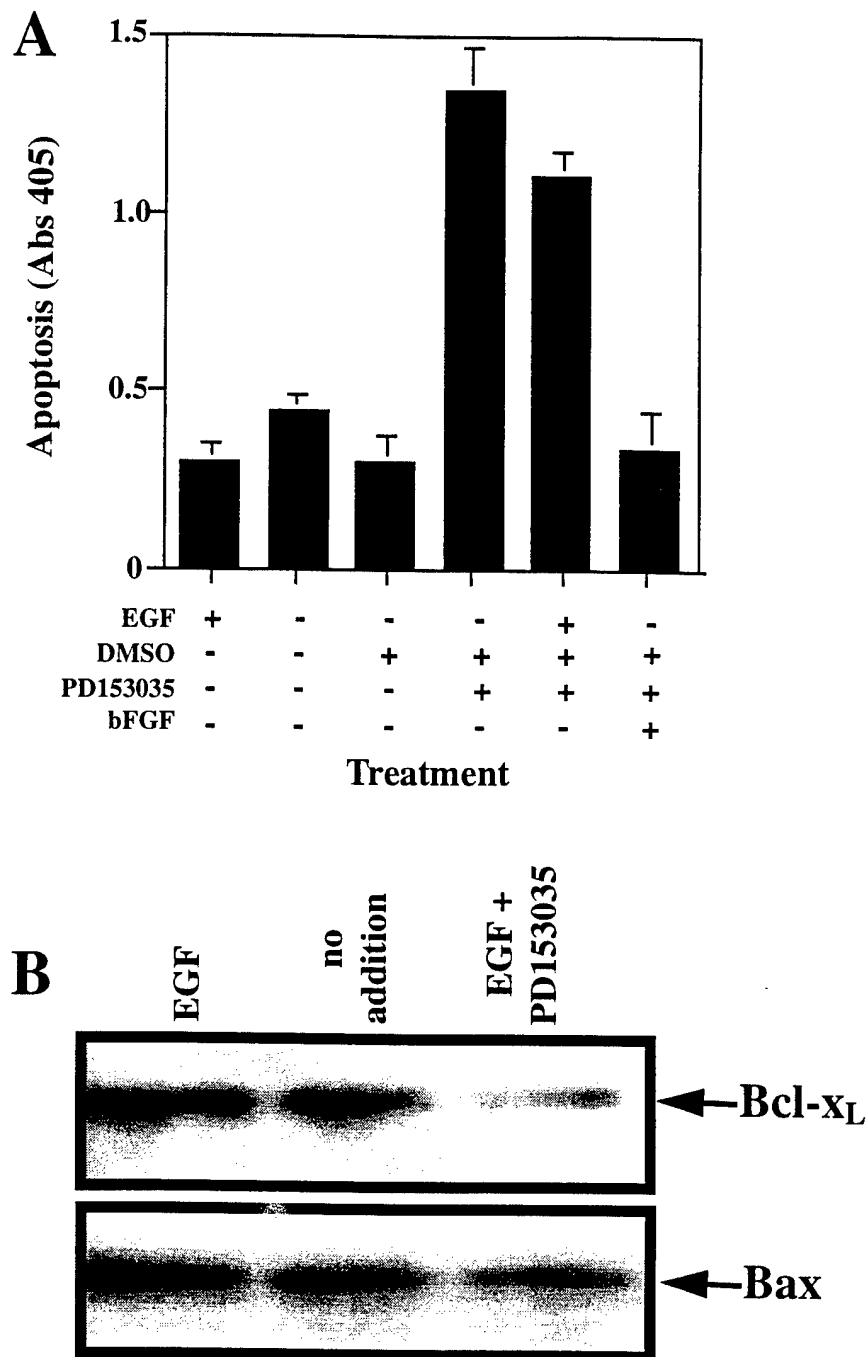


Figure 7: Effects of PD 153035 on TGF α /Myc#75 cells. *Panel A:* Induction of apoptosis in the TGF α /Myc#75 line by PD153035, a specific inhibitor of EGF receptor tyrosine kinase activity. Cells were incubated for 24 h with the indicated additions (10 ng/ml EGF or bFGF, 10 μ M PD153035, 1 μ l/ml DMSO as a control) and apoptosis was measured via ELISA assay. $n=4(\pm SE)$. *Panel B:* Cells were grown for 24 h with the indicated additions prior to preparation of whole cell lysates. 10 μ g of protein were analyzed by Bcl-x_L western blot as in Figure 5.

CONCLUSIONS

The results presented here provide evidence that uncontrolled c-myc expression in mammary epithelial cells can affect cell cycle regulation as well as cell survival. The growth rate of both mouse and human MECs was accelerated by c-myc overexpression. Based on our observations in the A1N4 model system, the difference in doubling time appears to be due to a shortened G_1 phase, as A1N4-myc cells reach S phase more quickly than parental cells when released from a G_1 growth arrest. In Figure 8, a model is proposed to explain the effects of constitutive c-myc expression on passage through the G_1 phase of the cell cycle. We suggest that Myc promotes the expression of cyclin E while suppressing the level of p27 cdk inhibitor, leading to increased cdk2 kinase activity. However, the mechanism by which Myc targets those two proteins is not clear and may be indirect. The primary function(s) of Myc is thought to be dependent on its ability to act as either a transcriptional activator or repressor, but a consensus binding sequence for Myc/Max has not been identified in the cyclin E promoter, and p27 levels are regulated at the post-transcriptional stage (27). That suggests that Myc is somehow activating other pathways which lead to altered expression of the two proteins in question. In contrast to cyclin E and p27, Cdc25A is a direct transcriptional target of Myc (28). The phosphatase may therefore also be critical for the Myc-induced increase in cdk2 activity in this system and is currently being investigated (see future directions below).

The elevated cdk2 activity is postulated to induce hyperphosphorylation of the G_1 inhibitory protein Rb. In its hypophosphorylated state, Rb interacts with a variety of transcription factors such as E2F-DP1 heterodimers and negatively regulates their activities. As the inhibitor becomes progressively phosphorylated during G_1 phase progression, the transcription factors are released, enabling them to activate transcription of genes required for DNA synthesis. However, in our MEC system, hyperphosphorylation by itself is not sufficient for passage through the cell cycle, since the cells clearly require EGF in addition to c-myc

overexpression for growth. We have demonstrated that EGF is required for the induction of cyclin D1 expression, but certainly there could be many additional, essential targets of the multifaceted EGF signal transduction pathway.

Overall, the results may provide at least a partial explanation as to why Myc and EGF can cooperate to transform cells and similarly, why there is such a strong synergism between Myc and TGF α in mammary tumorigenesis, as demonstrated by transgenic mouse models. Myc overexpression, in conjunction with EGF receptor stimulation, forces the cells through G₁ at a faster rate, resulting in accelerated growth and thus an elevated S-phase fraction. If this phenotype also allows the cells to proliferate under conditions which would normally induce a G₁ arrest and block DNA replication, increased genetic instability may also be a logical endpoint, analogous to the phenomenon which is demonstrated for p53 mutations. That hypothesis is currently being investigated in our laboratory.

An additional explanation for the cooperation of Myc and EGF can be found in our results with the Myc#83 transgenic cell line. We have shown that EGF and TGF α can act as survival factors for MECs which overexpress c-myc, while the growth inhibitor TGF β promotes Myc-induced apoptosis, even in the presence of EGF. In Figure 9, a model is postulated to explain the ability of those growth factors to regulate the apoptotic pathway when c-myc is constitutively overexpressed in MECs. We propose that Myc stimulates the expression of the pro-apoptotic proteins Bax and p53. This is most likely a direct stimulation, since the promoters of both genes contain Myc E-box sequences. The Bax promoter region also contains a p53 recognition site, suggesting that Bax expression may be additionally stimulated by p53 itself. The constitutive expression of Myc, and thus Bax and p53, make the cells highly susceptible to apoptosis unless a survival factor such as EGF is present to elevate levels of the survival promoting Bcl-x_L protein. The inhibitory TGF β signal is apparently dominant to that of EGF since it blocks the ability of the growth factor to elevate Bcl-x_L levels and therefore promotes Myc-induced apoptosis. However, the mechanism by which EGF modulates Bcl-x_L protein levels and the point at which TGF β interferes with that modulation are not known.

Previous results from our laboratory demonstrated that EGF and Myc could cooperate in bitransgenic mice to induce mammary tumors (12). Taken together, the results presented here may provide a mechanism for that strong synergism. Myc overexpression leads to elevated cdk2 activity and, in combination with EGF, promotes accelerated passage through the G₁ phase of the cell cycle. In addition, EGF acts as a survival factor for Myc-overexpressing cells by increasing Bcl-x_L protein levels.

Future Directions

We feel that the following experiments are essential to complete the aims as discussed in our previous report, as well as for publication of the final manuscripts for this project:

- 1) The models described here overexpress c-myc through constitutively active promoters (MMLV LTR and MMTV LTR). It would therefore be very informative to transfect cells with a regulatable construct, such as Myc-ER (in the estrogen receptor negative A1N4 cells) or a Tet system (29, 30). An inducible system would allow a more direct observation of the effects of inappropriate c-myc expression under a variety of conditions. It would also eliminate any selective pressure (either positive or negative) and the concomitant genotypic and/or phenotypic changes in the transfected cells.
- 2) As previously mentioned, Cdc25A is a strong candidate for a direct target of Myc which could promote both proliferation and apoptosis. Like Myc, Cdc25A expression is tightly regulated and correlates with the proliferative status of the cell (28). It can also function as a transforming oncogene in cooperation with activated Ha-ras (similar to Myc) or loss of Rb (31). The phosphatase clearly could be critical for cell cycle progression, since cdks are not active unless their inhibitory phosphates are removed by Cdc25. A role for Cdc25 in apoptosis is not so obvious, but its overexpression in serum starved cells leads to apoptotic cell death, similar to c-myc overexpression. Furthermore, oligonucleotides antisense to cdc25A can block Myc-induced apoptosis (28). Expression and activity of Cdc25 will therefore be examined in our MEC systems.

3) Since we have identified several cell cycle regulators which appear to promote growth in c-myc-overexpressing MECs, the logical next step would be to determine whether those proteins are essential for the accelerated growth rate of such cells. For example, transfection of the A1N4-myc cells with an inducible p27 expression vector or cyclin E antisense construct may provide insight as to whether altered regulation of those two proteins is critical for the observed phenotype. Such an experiment could also identify the time point in the cell cycle at which they are required. Conversely, inhibition of p27 expression or activity, or overexpression of cyclin E in the parental A1N4 cells would theoretically lead to increased cdk2 activity and thus Rb phosphorylation levels, similar to the A1N4-myc cells.

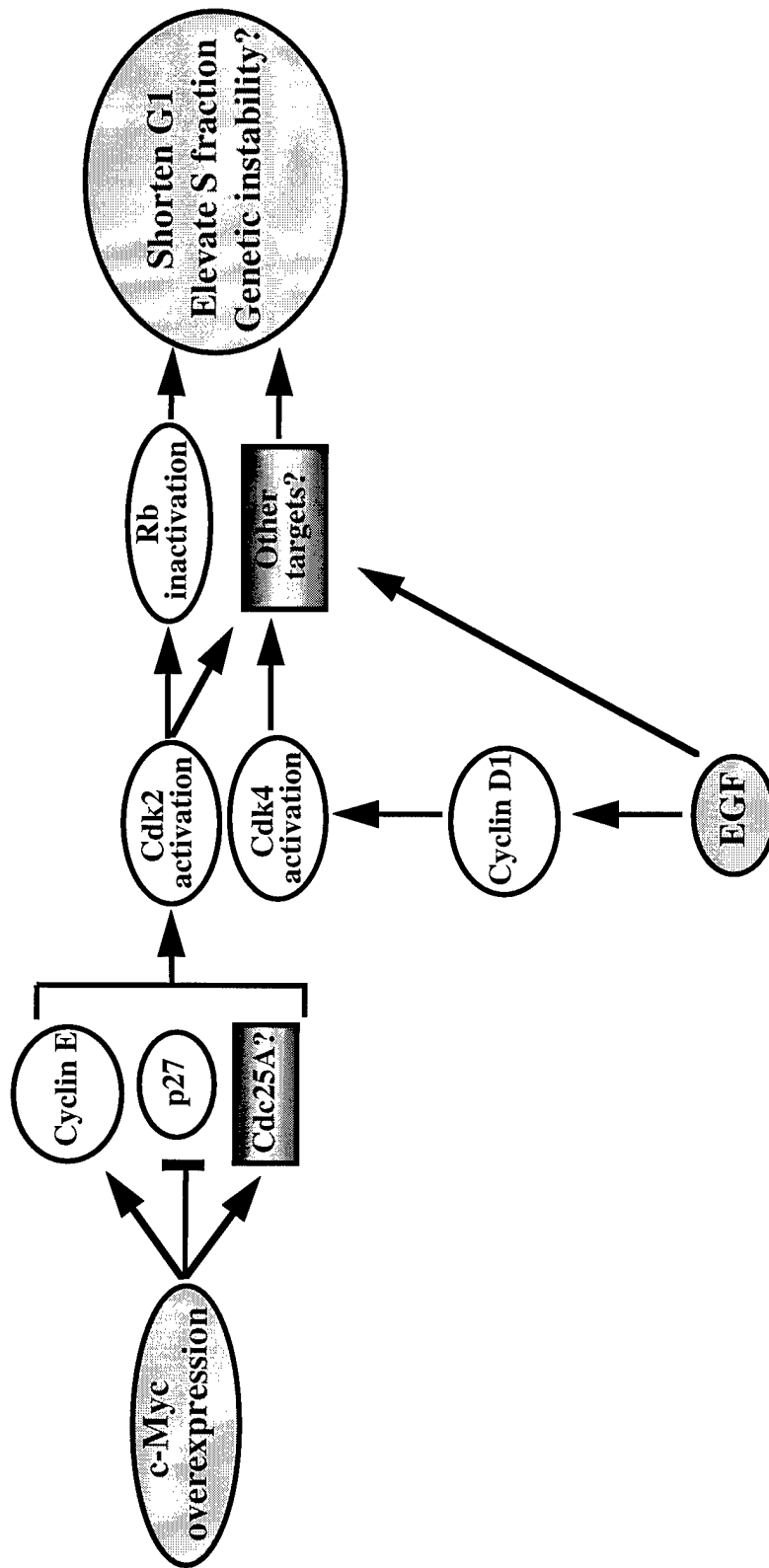


Figure 8: Proposed model for the effects of c-myc overexpression on cell cycle regulation in MECs. Myc induces cdk2 activation, *via* increased cyclin E expression and decreased p27 expression, which in turn can alter the phosphorylation state of Rb. Myc may also induce Cdc25A expression, which would further stimulate cdk2 activity. EGF is required for cyclin D1 expression. Additional targets of the EGF receptor signaling pathways may also be necessary for progression into S phase. The combination of c-myc overexpression and EGF signaling leads to accelerated proliferation and may therefore promote genetic instability.

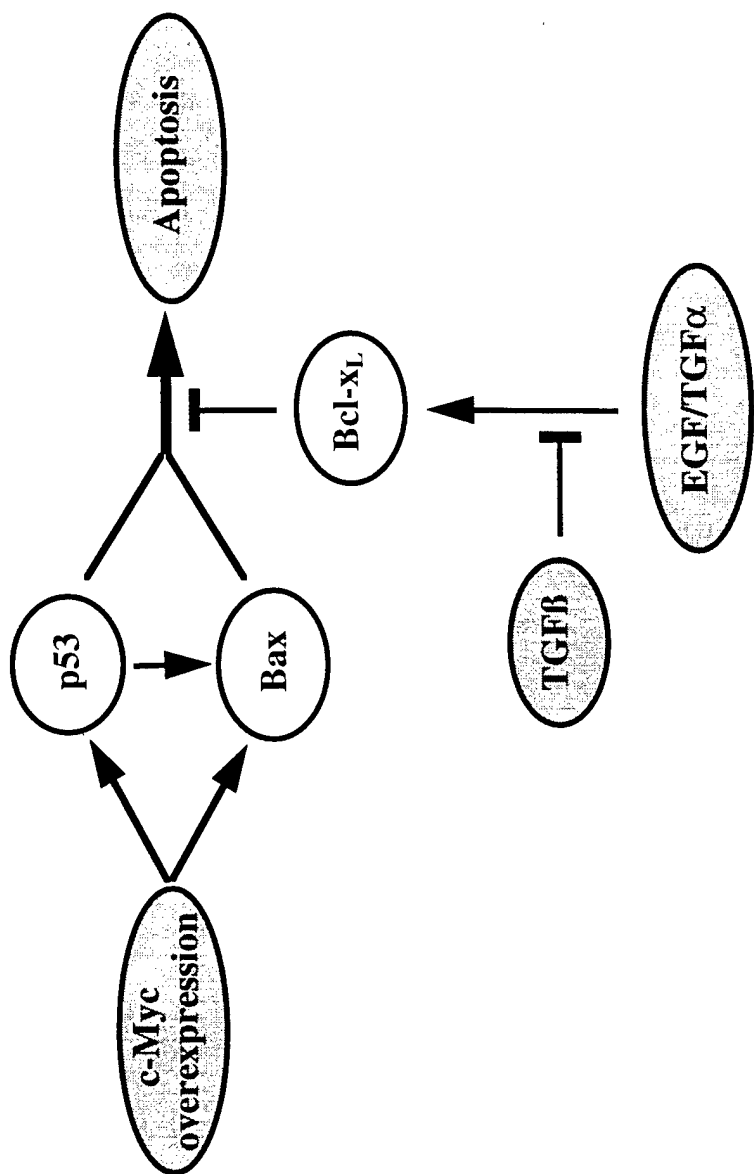


Figure 9: Proposed model for apoptosis induction by c-myc overexpression in MECs. Myc is postulated to stimulate the expression of the pro-apoptotic regulators p53 and Bax. p53 can itself also induce Bax expression. Apoptosis ensues unless a growth/survival factor such as EGF or TGF α is present to induce expression of the anti-apoptotic regulator Bcl-x_L. The growth inhibitor TGF β can block the effects of EGF, thereby promoting apoptosis.

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APPENDIX A

Manuscript in Press, Biochem. Biophys. Res. Comm.

ROLE FOR BCL-X_L IN THE REGULATION OF APOPTOSIS BY EGF and TGF β 1 IN c-MYC OVEREXPRESSING MAMMARY EPITHELIAL CELLS

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We previously showed that TGF α synergizes with c-myc in mammary tumorigenesis through inhibition of Myc-induced apoptosis. We therefore examined the effects of growth factors on apoptosis induction in several cell lines from MMTV-*myc* mammary tumors. When EGF was withdrawn or TGF β 1 was added, cells became apoptotic after 15 h (by ELISA and morphology). Northern and Western analysis revealed high levels of Bax and p53, and low or undetectable levels of Bcl-2 and Bcl-x_s under all treatment conditions. In contrast, Bcl-x_L expression was highest in the presence of EGF or TGF α , with a significant reduction upon removal of EGF or exposure to TGF β . In mouse mammary tumors, the relative Bcl-x_L/Bax ratio was higher in TGF α /Myc double transgenics than in Myc single transgenics, in agreement with the *in vitro* data. Our results suggest a role for Bcl-x_L in the regulation of apoptosis by EGF and TGF β in mammary epithelial cells.

The proto-oncogene *c-myc* encodes a transcription factor which forms a heterodimer with Max (1). Although the Myc/Max targets are not well defined, Myc is believed to have an important regulatory function in cell proliferation. Myc expression is tightly regulated and correlated with the proliferative state of the cell. Reduced *c-myc* levels due to disruption of one allele results in a lengthened G1 phase (2), while inhibition of *c-myc* expression blocks cell cycle progression and leads to G1 arrest (3). Conversely, cells which constitutively express *c-myc* cannot arrest in G1 and thus continue to proliferate or undergo apoptosis (4).

Activation of *c-myc* is thought to play a role in the development of breast cancer since it is commonly amplified and/or overexpressed in human breast tumors (5, 6). *c-myc* amplification is associated with a high proliferation index in mammary tumors and is correlated with poor prognosis. In addition, Myc confers tumorigenicity when overexpressed in the mammary gland of transgenic mice (7). Recent results indicate that overexpression of both *c-myc* and TGF α cooperate strongly in mammary tumorigenesis (8, 9). The contribution of TGF α may be due, at least in part, to the suppression of myc-induced apoptosis (10).

Apoptosis is regulated by the *bcl* family of proteins which either promote or inhibit cell death (11). The death suppressor Bcl-2, initially recognized for its role in lymphoma, has since been found to be highly expressed in a variety of tumors (12). When overexpressed, Bcl-2 can protect cells from many apoptotic signals, including unregulated myc expression (4, 11, 12). Bcl-x is a unique family member in that the mRNA can be alternately spliced to produce 2 different proteins: a death suppressor (Bcl-x_L) and a death inducer (Bcl-x_S). The suppressive activity of Bcl-2 and Bcl-x_L can be modulated by death inducers such as Bax, a family member which forms heterodimers with the two former proteins. The ratio of Bcl inducers to suppressors determines the fate of the cell (11). Bcl-x and Bax are both expressed in breast tissue and play an important role in the normal apoptotic process of mammary gland involution (13, 14).

The tumor suppressor p53 has also been implicated in the regulation of apoptosis and, like Myc, can regulate growth as well as death. Expression of p53 leads to cell cycle arrest or apoptosis, depending on the cell type and environment. In many, but not all systems, p53 activity is required for apoptosis. In the mammary gland, apoptosis can occur in the absence of p53 (14, 15), but it is not known whether myc-induced apoptosis requires wild type p53 in that cell type.

For this study, we wished to investigate the regulation of apoptosis in mammary epithelial cells which overexpress *c-myc*. Mammary tumors which arise in MMTV-myc transgenic mice, as well as epithelial cell lines derived from such tumors, both show a high propensity to undergo apoptosis (10). However, mammary tumors and cell lines which express both Myc and TGF α exhibit very low levels of apoptosis and therefore grow much faster *in vitro* and *in vivo*. Furthermore, apoptosis in the Myc single transgenic cell lines can be inhibited by exogenous TGF α or EGF and accelerated by the growth inhibitor TGF β . We therefore examined expression of apoptotic pathway genes in the presence or absence of those growth factors.

MATERIALS AND METHODS

Transgenic Mice. The TGF α transgenic mice (MT100) were provided by Dr. Glenn Merlino (NIH, Bethesda, MD). The c-Myc mice (MMTV-c-myc M) were developed by Dr. Philip Leder (Harvard Medical School, Boston, MA) and obtained from Charles River Breeding through a breeding license with DuPont. Double transgenic mice were generated as described previously by mating the TGF α strain to the c-Myc strain (8). Mammary tumors were excised and frozen at -70°C as they spontaneously arose in each strain.

Cell Lines. The cell lines Myc#83, Myc#7, and Myc#9 were established from mammary tumors of single transgenic mice as previously described (10). The cells were routinely grown in IMEM (Gibco-BRL) containing 2.5% FCS, 10 ng/ml EGF (Upstate Biotechnology Incorporated) and 5 μ g/ml insulin (Biofluids).

Cell Death ELISA. Cytoplasmic nucleosomal DNA fragments were detected using an apoptotic cell death ELISA (Boehringer Mannheim) with antibodies directed against histones and DNA, as previously described (66). Cells were plated in 12-well plates (6.7×10^4 cells/well) and were treated for 3-48 hours prior to lysis with the following growth factors: EGF (10 ng/ml), TGF β 1 (100 pM, R & D Systems), or EGF plus TGF β .

Northern analysis. Cells were plated (5×10^5 cells/ 60 mm plate) and treated as above for 24 h. Total RNA was harvested by the guanidine thiocyanate-acid phenol method. Total RNA (15 μ g) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (Amersham). Blots were sequentially hybridized overnight with the following 32 P-labeled, random-primed murine probes: bcl-2 (nt 1635-1945), bcl-x_{L+S} (mRNA nt 110-394), bax (mRNA nt 138-389), and p53 (nt 97-1407).

RT-PCR. The relative amounts of bcl-x_L and bcl-x_S mRNA were measured by an RT-PCR assay. One μ g RNA from each sample was reverse transcribed with random primers and the cDNA for bcl-x_{L+S} was amplified for 31 cycles using a pair of primers that amplify the nucleotide sequence containing the region differentially spliced in the bcl-x_L and bcl-x_S mRNAs. 5' primer (mRNA nt 466-488): 5' -GCG CGG GAG GTG ATT CCC ATG GC-3'; 3' primer (nt 891-870): 5'-CAT GCC CGT CAG GAA CCA GCG G-3'. PCR products were fractionated on a 2% agarose gel and transferred to a nylon membrane which was sequentially hybridized with a 32 P-labeled random-primed probe for bcl-x_L (Bcl-x mRNA nt 466-891) and an oligonucleotide specific for the splice site within the 237-bp bcl-x_S product (5'-CAG AGC TTT GAG CAG GAC ACT TTT GTG G-3').

Western Analysis. Cells were plated (1.3×10^6 cells/100 mm plate) and treated as above for 24 h before lysis in RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). Frozen mouse mammary tumors were pulverized in liquid nitrogen and homogenized in Tris-SDS (10 mM, pH 7.4, 1%). Twenty μ g of protein were separated on 14% SDS-PAGE gels (or 10% for p53) and transferred to nitrocellulose. Blots were blocked with 5% milk in Tris-buffered saline with Tween-20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween-20) for 1 h and then incubated in TBST with 1% BSA and the following antibodies (diluted 1/400): Bcl-x_{L+S} (S-18), Bax (N-20), Bcl-2 (N-19) (Santa Cruz Biotechnology); or p53 (Ab-1, Oncogene Sciences). Proteins were visualized with an HRP-linked second antibody (1/500 in TBST with 1% BSA) and a chemiluminescent detection system (Pierce).

RESULTS

Three mammary epithelial cell (MEC) lines derived from tumors of MMTV-myc mice were tested for their apoptotic response to EGF and TGF β . Southern analysis demonstrated that the cell lines retained the myc transgene, and expression *in vitro* was confirmed by Northern and Western blots (not shown). Cells were treated for 24 h before preparing cytoplasmic lysates for assessing apoptosis via histone-DNA ELISA. For each cell line, the occurrence of apoptosis was lowest in the presence of EGF (Figure 1A). The level of apoptosis increased dramatically following EGF withdrawal or exposure to TGF β . However, there was no apparent synergism for apoptosis induction between addition of TGF β and removal of EGF.

The time course of apoptosis induction was assessed in the Myc#83 cells. At all time points, cells treated with EGF showed very low, basal levels of apoptosis (Figure 1B). In contrast, cells deprived of EGF or treated with TGF β showed a very similar temporal pattern of cell death. They began to exhibit cytoplasmic histone-DNA complexes at about 15 hours after treatment and achieved maximal levels between 24 and 48 hours.

The ELISA results were confirmed by observing morphological changes in Myc#83 cells undergoing apoptosis (not shown). Cells grown with EGF appeared healthy and displayed many mitotic figures. In contrast, cells exposed to TGF β or deprived of EGF exhibited a very typical apoptotic morphology, with DNA condensation at the nuclear membrane and prominent apoptotic bodies. Furthermore, mitotic structures were rare under conditions which favored apoptosis.

Based on the results in Fig. 1B, a 24 h treatment period was chosen to assess changes in gene expression. Northern blots were sequentially hybridized with murine probes for bcl-2, bcl-x, bax, and p53 (Figure 2). Bcl-x showed the greatest variation in expression. Levels were highest in EGF treated cells and were significantly decreased following TGF β treatment or EGF withdrawal. Expression of bcl-2 was clearly much lower than bcl-x, but the pattern of expression was similar, with the strongest signal for cells grown in the presence of EGF. In contrast, bax RNA was easily detectable and was relatively uniform across treatments. Expression of p53 RNA was also consistent across treatments, with the notable exception of EGF deprivation (50% reduction).

RT-PCR was used to distinguish the long and short forms of bcl-x RNA (Figure 3). A single band corresponding to bcl-x_L was visible on ethidium bromide stained gels of the PCR products. When the DNA was transferred to nylon and hybridized with a random-primed probe, a strong signal for bcl-x_L was observed. Hybridization with a bcl-x_S specific oligonucleotide revealed a much weaker band for the short form, with little fluctuation among treatments. The results suggest that the bcl-x signal observed by Northern analysis was largely due to expression of bcl-x_L rather than bcl-x_S.

Western blots were used to examine expression at the protein level (Figure 4). Bcl-x_L protein levels varied dramatically, with highest expression in EGF-treated cells. Bcl-x_L expression was similar in EGF- and TGF α -treated cells (not shown). Following TGF β treatment or EGF withdrawal, there was an 80% reduction in Bcl-x_L protein. Analysis of lysates prepared at various time points indicated that the decrease in Bcl-x_L protein preceded the onset of DNA degradation (not shown). Neither Bcl-x_S nor Bcl-2 could be detected by Western analysis. Bax and p53 protein levels were relatively high and showed little variation across treatments.

The relative ratios of Bcl-x_L to Bax were compared in mammary tumors from single (MMTV-myc) or double (MMTV-myc X MT-TGF α) transgenic mice. Bcl-x_L and Bax were detected by Western analysis and relative ratios of the signal intensity for the two proteins were calculated from the values obtained by densitometry (Figure 5). Lysates from Myc single transgenic tumors consistently showed a relative ratio of Bcl-x_L to Bax of approximately 0.5 (+/-

0.06). Ratios for the double transgenic tumors were more variable, but were consistently higher than the single transgenics, with a mean value of 2.9 (+/- 1.0). Bcl-2 was not detected in the tumor lysates.

DISCUSSION

Our results suggest a role for Bcl-x_L in growth factor-mediated regulation of apoptosis in MECs which overexpress c-Myc. Bcl-x_L expression showed the greatest variation in response to EGF and TGFβ. Bcl-x_L mRNA and protein levels were dramatically elevated in Myc#83 cells grown in the presence of EGF or TGFα compared to cells which had been deprived of EGF or treated with TGFβ. Consistent with the *in vitro* results, the relative ratio of Bcl-x_L to Bax was higher *in vivo* in mouse mammary tumors which expressed TGFα and Myc together compared to those which overexpressed Myc alone.

The data indicate that MECs which overexpress c-Myc are dependent on EGF receptor signaling for survival and growth. We and others have recently demonstrated that EGF and TGFα can act as survival factors for mammary epithelial cells *in vitro* (16) and *in vivo* (10, 17). However, in the aforementioned *in vitro* study (16), much harsher conditions were used to induce apoptosis in MECs than in our study (high cell density combined with serum deprivation rather than simple withdrawal of EGF from subconfluent cells in the presence of 2.5% FCS and 10 μg/ml insulin). Many MEC lines, both human and mouse, are dependent on EGF for growth, but deprivation of EGF under normal culture conditions results in reversible growth arrest rather than apoptosis (18; personal communication, Dr. Daniel Medina, Baylor College of Medicine, Houston, TX). Those results imply that Myc-overexpressing MECs are very susceptible to the induction of apoptosis and are especially dependent on EGF receptor pathway(s) for survival. Our cell lines therefore provide an excellent model for studying growth/survival factor regulation of Myc-induced apoptosis in MECs. The results presented here indicate that increased expression of Bcl-x_L may, at least in part, explain the mechanism by which EGF and TGFα function as survival factors.

The manner in which EGF regulates apoptosis in MECs may therefore be analogous to that observed in hematopoietic cells following cytokine withdrawal. Two recent reports implicate Bcl-x_L in the control of cell survival in normal activated T cells and in myeloid leukemia cells (19, 20). In the former study, expression of Bcl-x_L in activated T cells was significantly reduced following interleukin-2 withdrawal, while bax and bcl-2 levels did not change. The latter study showed a reduction of Bcl-x_L expression in leukemia cells following survival factor withdrawal.

Some hematopoietic cancer cells also undergo apoptosis in response to TGFβ, as in our MEC system. TGFβ induced apoptosis in leukemia cells with a concomitant decrease in Bcl-2 expression, but no change in Bax expression (21), while in lymphoma B cells, apoptosis induction by TGFβ was not accompanied by changes in Bcl-2 expression (22), suggesting that regulation of cell death by TGFβ may be cell type specific. Bcl-x_L expression was not examined in those studies.

Increased TGF β expression has been observed in MECs which have been stimulated to undergo apoptosis (23, 24). However, it was not determined whether TGF β secretion was required for apoptosis induction. *In vitro*, TGF β has previously been shown to inhibit growth of MECs (25, 26), but it has not been reported to induce apoptosis. In contrast, overexpression of TGF β 1 *in vivo* has been associated with increased occurrence of apoptosis in normal MECs (27). Since the levels of growth/survival factors such as EGF and estrogen are relatively high in the mammary gland during pregnancy, the effects of TGF β are presumed to be dominant over those of the positive survival signals, analogous to our *in vitro* observations. Our results suggest that TGF β may block the induction of Bcl-x_L expression by survival factors like EGF.

In contrast to Bcl-x_L, expression of Bax and p53 were relatively constant in the MECs across the various treatments examined. Since c-Myc can transactivate the p53 promoter (28), and the *bax* gene promoter also contains a putative c-Myc response element (29), those two genes may be continuously activated when Myc is constitutively expressed. That could explain the propensity of Myc-overexpressing cells to undergo apoptosis when challenged with a negative growth signal. High levels of Bax may determine the "set point" of the cells so that any decrease in the protective Bcl family members due to removal of growth/survival signals will push cells toward apoptosis. Myc may activate the p53 and Bax pathways as a safeguard to prevent the survival of cells with oncogenic activation. The mechanism by which Myc triggers cell death is not universal however, since the induction of apoptosis by myc overexpression is dependent on wild type p53 in some, but not all systems (4, 30). The role of p53 in our MEC system is currently being examined.

Bcl-2 and Bcl-x_S apparently do not play a significant role in the MEC system examined here. The RNA levels for both were quite low and the proteins were undetectable on Western blots. Bcl-x_L and Bcl-2 regulate a common pathway (31), but the tissue specific expression patterns of the two genes do not always overlap (32), perhaps allowing for cell specific responses to different stimuli. For example, in the human breast, Bcl-2 is not expressed in functionally differentiated secretory cells, although it can be detected in non-secretory epithelial cells (33). Alternatively, the low Bcl-2 levels may be secondary to the expression of p53 in Myc#83 cells. The 5' untranslated region of the *bcl-2* gene contains a p53-dependent negative response element (34) and p53 can down-regulate *bcl-2* expression in human breast cancer cells (35).

In summary, we have demonstrated a potential role for the cell survival-promoting protein Bcl-x_L in MEC apoptosis driven by c-Myc overexpression. Myc activation is common in breast cancer, but it is well documented that Myc overexpression alone is insufficient for transformation. Since Myc can induce apoptosis as well as growth, it is likely that secondary events will block cell death, thereby allowing the stimulatory effects of Myc to predominate. We suggest that changes in Bcl-x_L expression, either directly *via* genetic alteration, or indirectly *via* increased production of survival factors, may promote tumorigenesis of cells which overexpress Myc.

Acknowledgments

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FIGURE LEGENDS

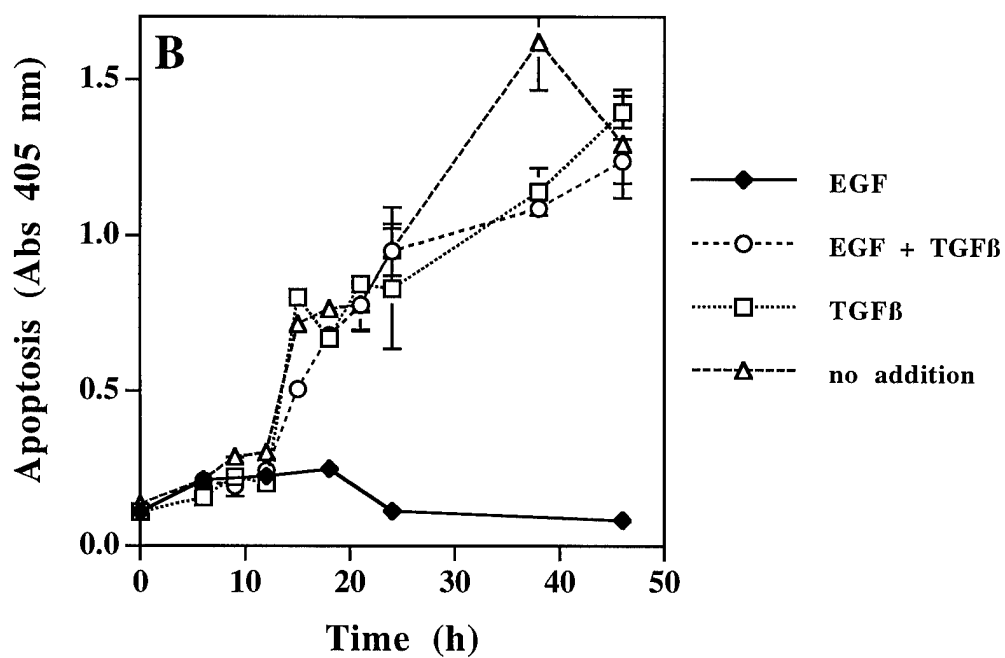
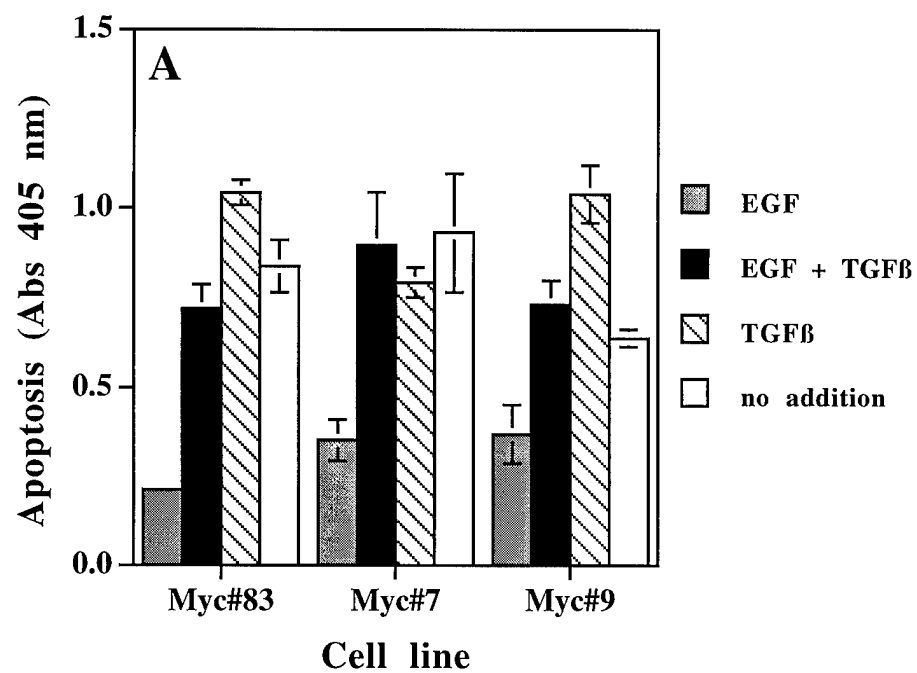
- Figure 1:** Apoptosis in MECs which overexpress c-myc. **A:** Three cell lines derived from mammary tumors of MMTV-myc transgenic mice were treated for 24 h with: EGF (10 ng/ml), TGF β 1 (100 pM), EGF + TGF β , or no addition. Apoptotic DNA fragments were detected in cytoplasmic lysates via histone-DNA ELISA. n=2 (+/-SE). **B:** Time course of apoptosis induction in Myc#83 cells. Cells were treated as in A and lysates were harvested at three hour intervals for ELISA. n=4 (+/-SE).
- Figure 2:** Northern Analysis. Myc#83 cells were treated for 24 h as in Fig. 1A before harvesting total RNA. **A:** Representative results. Northern blots were sequentially hybridized with random-primed probes for mouse bcl-2, bcl-x, bax, and p53. 28S and 18S ribosomal RNA bands are shown as a loading standard. Autoradiography exposure times were as follows: bcl-2, 7 d; bcl-x_{L+S}, 5 d; bax, 3 d; p53, 2 d. **B:** Cumulative data. The mean band intensity for untreated cells was assigned a value of 1 and all other values were calculated as a relative increase or decrease. n=8 (+/-SE).
- Figure 3:** Bcl-x_(L,S) RT-PCR. RNA samples were reverse-transcribed and then amplified by PCR. The PCR products were separated on an agarose gel and transferred to nylon for Southern analysis. **A:** The blot was hybridized with a random-primed probe (Bcl-x mRNA nt 466-891). (3 h exposure). **B:** The blot was hybridized with an oligonucleotide probe specific for the short form of bcl-x. (20 h exposure).
- Figure 4:** Western analysis of Myc#83 cells. Cells were treated for 24 h as in Fig 1A before preparing total cell lysates. 20 μ g of protein were separated by SDS-PAGE and transferred to nitrocellulose. **A:** Representative results. Individual blots were incubated with antibodies against Bcl-x, Bax, and p53. **B:** Cumulative data. The mean band intensity for untreated cells was assigned a value of 1 and all other values were calculated as a relative increase or decrease. n=6 (+/-SE).
- Figure 5:** Western analysis of mammary tumors from single Myc and double Myc/TGF α transgenic mice. Lysates were prepared from frozen tumors and 20 μ g of protein were separated on 14% polyacrylamide gels. The blot was probed first with an antibody against Bcl-x and then stripped and re-probed with Bax antibody. Relative ratios were calculated from the band intensities as measured by densitometry.

For Figures 2-5, see body of the report (Fig 4-6)

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Cooperation of TGF α and c-Myc in mouse mammary tumorigenesis: coordinated stimulation of growth and suppression of apoptosis

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We have previously shown that TGF α and c-Myc interact in a strong, synergistic fashion to induce mammary gland tumors in double transgenic mice. Here we show this interaction can be explained, at least in part, by a cooperative growth stimulus by the two proteins, and by TGF α -mediated inhibition of c-Myc-induced apoptosis. We initially compared rapidly progressing mammary tumors from double transgenic mice to long latency tumors from single transgenic mice and observed a striking difference in the occurrence of apoptosis among the three groups. Tumors exhibiting apoptosis were derived exclusively from mice that expressed the c-myc transgene in the absence of the TGF α transgene, indicating that TGF α might protect c-Myc-overexpressing cells from programmed cell death. Cell lines were derived from single and double transgenic mammary tumors to examine further the mechanism underlying the cooperative interaction between the two gene products. In accordance with our *in vivo* data, apoptosis was only detected when the c-myc transgene was expressed without the TGF α transgene. Furthermore, exogenous addition of TGF α inhibited apoptosis in cells overexpressing c-Myc alone. In addition, tumor-derived cells that overexpressed both TGF α and c-Myc exhibited faster growth rates *in vitro* and *in vivo* and were less sensitive to the inhibitory effects of TGF β *in vitro* compared to cell lines expressing only one of the transgenes. Based on our findings we propose that TGF α acts both as a proliferative and a survival factor for c-Myc-expressing tumor cells. Our results indicate that TGF α and c-Myc cooperate in tumorigenesis via a dual mechanism: TGF α can inhibit c-Myc-induced apoptosis and both proteins provide a growth stimulus.

Keywords: c-Myc; TGF α ; apoptosis; mammary tumorigenesis

Introduction

It is well documented that overexpression of the proto-oncogene c-myc can induce proliferation, transforma-

tion, and apoptosis (Askew *et al.*, 1991; Evan *et al.*, 1992; Kato and Dang, 1992; Khazaie *et al.*, 1991; Marcu *et al.*, 1992; Meichle *et al.*, 1992; Telang *et al.*, 1990; Valverius *et al.*, 1990). It has also been reported by a number of investigators that c-Myc can cooperate with growth factors such as transforming growth factor alpha (TGF α) or epidermal growth factor (EGF) to promote a transformed phenotype *in vitro* (Khazaie *et al.*, 1991; Stern *et al.*, 1986; Telang *et al.*, 1990; Valverius *et al.*, 1990). We and others have recently shown that c-Myc and TGF α synergize in an extremely strong way to induce mouse mammary gland tumors in transgenic mice *in vivo* as well (Amundadottir *et al.*, 1995; Sandgren *et al.*, 1995). In order to understand the mechanisms responsible for this interaction, we were interested in examining proliferation, anchorage independent growth, and apoptosis as possible points of interaction between TGF α and c-Myc that may enhance tumorigenesis in the mammary gland.

Both gene products have been implicated in the genesis of many human cancers, including breast tumors. The c-myc gene is frequently found amplified and/or overexpressed in human breast cancer (Bonilla *et al.*, 1988; Escot *et al.*, 1986; Garcia *et al.*, 1989; Mariani-Costantini *et al.*, 1988). Although TGF α is not amplified at the gene level in human breast cancer, its expression (and that of other EGF family members) is frequently increased compared to the normal gland (Arteaga *et al.*, 1988; Bates *et al.*, 1988; Derynck *et al.*, 1987; Perroteau *et al.*, 1986; Travers, 1988). In addition, various groups have reported a tumorigenic action of these genes when overexpressed in the mammary gland of transgenic mice (Jhappan *et al.*, 1990; Leder *et al.*, 1986; Matsui *et al.*, 1990; Sandgren *et al.*, 1990; Schoenenberger *et al.*, 1988; Steward *et al.*, 1984).

Apoptosis is an active process whereby the cell is programmed to carry out a series of events that eventually lead to its auto-destruction. When apoptosis is initiated, cells undergo various biochemical and morphological changes which result in the degradation of genomic DNA and fragmentation of the cell into apoptotic bodies (Bellamy *et al.*, 1995). Apoptosis occurs during development as well as in adult organisms and can be activated or inhibited by specific agents, such as hormones or growth factors (Schwartzman and Cidlowski, 1993). Inhibition of apoptosis can also contribute to tumorigenesis.

The c-Myc protein has been implicated in the regulation of apoptosis. When c-Myc expression is deregulated, cells are prone to enter an apoptotic pathway, depending on the cell environment (Askew *et al.*, 1991; Evan *et al.*, 1992). A number of survival factors which protect cells from c-Myc-mediated

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The first two authors (LT Amundadottir and SJ Nass) made equal contributions to the writing of this manuscript. RTA established and characterized the 3 cell lines in the study and was responsible for Figures 1, 2ab, 3ab, 5a and 6 while SJN contributed Figures 1, 2c, 3ac, 4, 5ab and 6.

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apoptosis have been identified (Askew *et al.*, 1991; Harrington *et al.*, 1994). In mouse fibroblasts for example, apoptosis induced by c-Myc was inhibited by various growth factors, such as insulin-like growth factors and PDGF, whereas EGF and bFGF were ineffective. In the current study, we show that TGF α can function as a survival factor for mammary epithelial cells which overexpress c-Myc. We observed that apoptosis occurred in mouse mammary gland tumors and in their derived epithelial cell lines only in cases where c-Myc was overexpressed in the absence of TGF α overexpression. Furthermore, when c-Myc overexpressing cells were treated with TGF α *in vitro*, apoptosis was greatly decreased. Our results suggest an explanation for the cooperative interaction between TGF α and c-Myc in tumorigenesis: both factors stimulate anchorage dependent proliferation and anchorage independent growth, and in addition, TGF α suppresses c-Myc-induced apoptosis.

Results

Programmed cell death occurs only in mammary gland tumors from c-myc single transgenic mice

To examine whether apoptosis was a factor in the cooperation between TGF α and c-Myc in tumorigenesis, we measured apoptosis in five mammary gland tumors from each of the three transgenic mouse strains (double transgenic TGF α /c-myc mice, and single transgenic TGF α and c-myc mice). *In situ* nick end-labeling of nucleosomal fragments by Klenow DNA polymerase I revealed that apoptosis was occurring in mammary gland tumors from c-myc transgenic animals and not in mammary tumors from single transgenic TGF α animals or double transgenic TGF α /c-myc animals. As shown in Figure 1c, only tumors from c-myc mice exhibited scattered cells with positive staining. When apoptosis was quantitated by counting apoptotic cells in 20 random fields of each tumor type (400 \times magnification) we observed that tumors from c-myc mice had 23.0 ± 2.8 apoptotic cells per field whereas tumors from TGF α and TGF α /c-myc animals had 0.3 ± 0.2 and 1.2 ± 0.5 labeled cells per field respectively.

Generation of cell lines from mammary gland tumors

We have generated three cell lines from mammary gland tumors arising in double and single transgenic mice. Our intent was to use them to verify our findings in mammary gland tumors *in vivo* and examine further the molecular mechanisms underlying the cooperation between TGF α and c-Myc. The following nonclonal cell lines were generated: TGF α /Myc#75 was derived from a tumor arising in a double transgenic virgin female (TGF α /c-myc animal number 75), Myc#83 was derived from a c-myc virgin single transgenic female (c-myc animal number 83) and TGF α #13 from a multiparous TGF α single transgenic female (TGF α animal number 13). Additional cell lines from tumors arising in c-myc and TGF α /c-myc animals have recently been isolated and were used where noted to confirm our findings with the first three cell lines.

Expression of cytokeratins and morphology of cell



Figure 1 Detection of apoptosis in mammary gland tumors from transgenic mice. Tumor sections were analysed by *in situ* nick end-labeling of DNA fragments. (a) shows a tumor from a double transgenic virgin TGF α /c-myc mouse, (b) a tumor from a single transgenic multiparous TGF α mouse and (c) a tumor from a single transgenic c-myc mouse. Note cytoplasmic staining in scattered cells of the tumor from a c-myc single transgenic animal (arrows, c) indicating DNA fragmentation

lines grown as subcutaneous tumors in nude mice was used to verify epithelial origins of the cell lines. The single transgenic cell lines TGF α #13 and Myc#83 were positive for keratin 14 at the mRNA level (not shown). TGF α /Myc#75 cells apparently did not express keratin 14, but positive immunofluorescent signal was observed in these cells with a pan-keratin antibody (not shown). All three lines also gave rise to tumors in nude mice that had a very distinct epithelial morphology. We therefore conclude that all three lines are epithelial, but that line TGF α /Myc#75 has probably lost expression of some of its keratins. This is not without precedent, since human breast

carcinoma cell lines, especially hormone independent lines, have been described to do the same (Sommers *et al.*, 1989, 1992).

Expression of the TGF α and c-myc transgenes was also assessed by Northern analysis (not shown). The single transgenic lines, TGF α #13 and Myc#83, expressed only the TGF α or c-myc transgenes, respectively. In contrast, the double transgenic cell line, TGF α /Myc#75, expressed both transgenes. Therefore, all three cell lines expressed the expected transgenes at the mRNA level. In addition, expression of the TGF α transgene was upregulated by ZnCl₂ and CdCl₂ as expected, since the TGF α transgene is expressed from the heavy metal-inducible metallothionein promoter (Jhappan *et al.*, 1990). The endogenous c-myc gene was downregulated in the two cell lines expressing the c-myc transgene (TGF α /Myc#75 and Myc#83), consistent with a negative autoregulation of the c-Myc protein on its own promoter, as has been described previously (Penn *et al.*, 1990).

In order to assess ploidy of the cell lines, cells were stained with propidium iodide and analysed by FACS. Each cell line was tested at two or three different timepoints (between passages 6 and 31). Only the double transgenic line TGF α /Myc#75 was found to be aneuploid. It was tetraploid at all three timepoints tested. In contrast, the two single transgenic lines were diploid at the time points tested (not shown).

Apoptosis occurs in c-Myc overexpressing mammary tumor cells in vitro

Tumor derived cell lines were tested for their ability to undergo apoptosis by two independent methods. For the first method, cytoplasmic DNA fragments were isolated and run on agarose gels. In this assay, the Myc#83 line was positive with a characteristic nucleosomal ladder whereas both TGF α #13 and TGF α /Myc#75 were negative (Figure 2a). Those results were confirmed by an ELISA apoptosis assay that is based on detecting histone-associated DNA in cytoplasmic cell lysates via a peroxidase catalyzed color change (A₄₀₅). CEM cells (a T cell leukemia cell line) treated with 10⁻⁷ M dexamethasone served as a positive control (Catchpoole and Stewart, 1993; not shown). The Myc#83 line showed a high degree of apoptosis, whereas the other two had levels close to background (Figure 2b). Five additional cell lines derived from Myc-single transgenic tumors also showed a propensity to undergo apoptosis as determined by the apoptosis ELISA (not shown). The appearance of apoptotic Myc#83 cells under conditions of EGF deprivation or TGF β 1 treatment was further confirmed by observing morphological changes which are characteristic of apoptosis (Figure 2c). The cells displayed prominent apoptotic bodies with concomitant reduction of cytoplasm and altered nuclear morphology.

When the Myc#83 line was treated with the growth factors TGF α , EGF, IGF-I or bFGF, apoptosis was inhibited up to 75% as measured by the ELISA assay (Figure 3a). In contrast, treatment with TGF β 1 resulted in elevated levels of apoptotic DNA, even in the presence of EGF. The effects of both TGF α and TGF β 1 on apoptosis were concentration dependent (Figure 3b and c), with maximal responses at 10 ng/ml and 100 pM, respectively.

Additional evidence for the importance of the TGF α /EGF receptor system was provided by using a synthetic inhibitor of EGF receptor tyrosine kinase activity (PD153035). PD153035 has been shown to

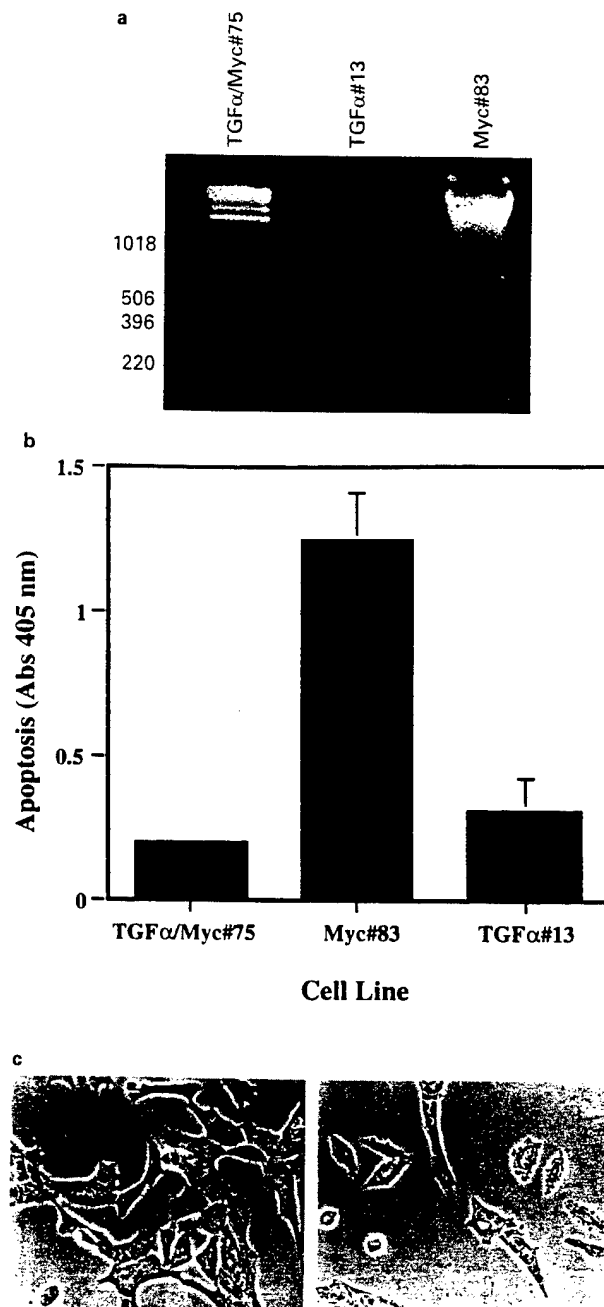


Figure 2 Detection of apoptosis in tumor-derived cell lines. (a): Nucleosomal ladder. DNA was isolated from cytoplasmic fractions of transgenic tumor cell lines and run on a 1.8% agarose gel which was stained with ethidium bromide. Note characteristic nucleosomal DNA with a size interval of approximately 180 bp. (b): ELISA-apoptosis assay. Apoptotic DNA fragments were detected in cytoplasmic lysates via a histone-DNA ELISA. Peroxidase substrate conversion was quantitated by measuring absorbance at 405 nm. (c): Morphological changes in apoptotic Myc#83 cells. Cells on the left side were grown in media containing EGF. The right side is representative of cells which have become apoptotic via EGF deprivation or TGF β 1 treatment, for 24 h. Note the characteristic reduction in cell size due to cytoplasmic blebbing (apoptotic bodies)

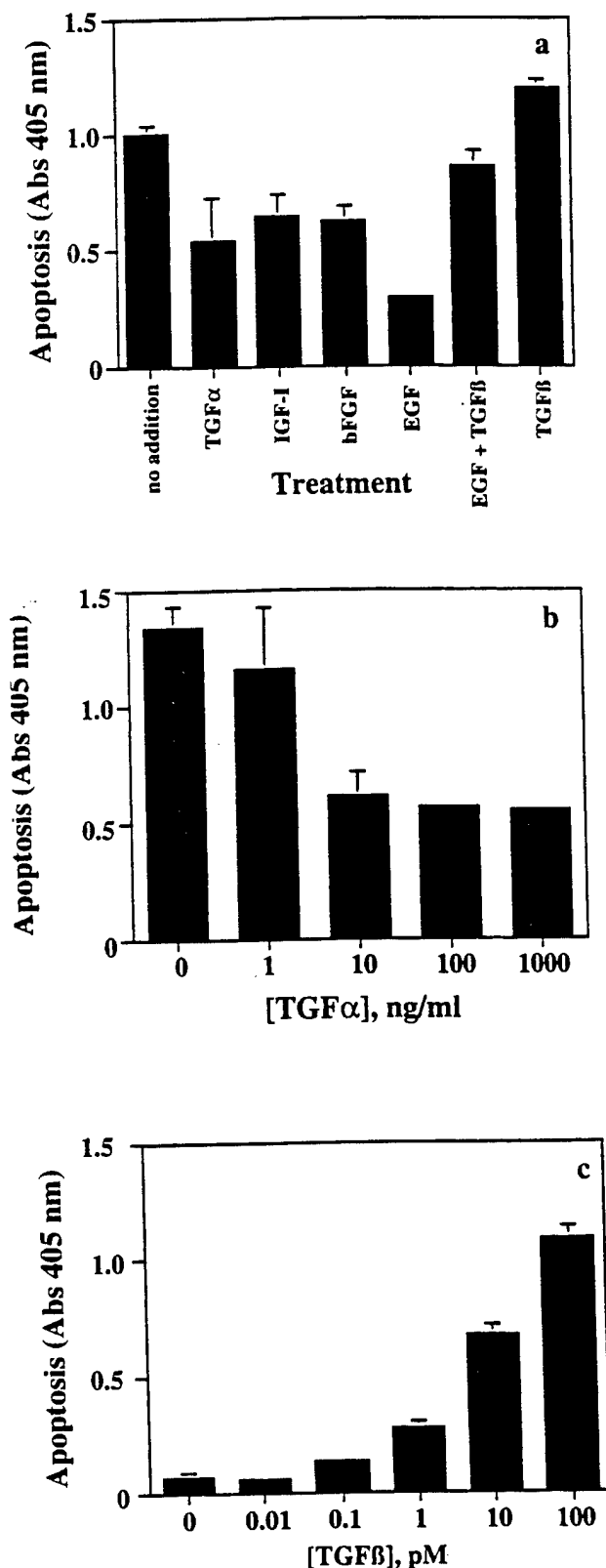


Figure 3 Effects of growth factors on apoptosis in the Myc#83 line. (a) shows the effects of TGF α (5 ng/ml), IGF-I (50 mM), bFGF (8 ng/ml), EGF (10 ng/ml), TGF β 1 (100 pM) or EGF + TGF β on apoptosis. (b) shows that the inhibitory effect of TGF α on apoptosis in the Myc#83 cell line is concentration dependent. (c) demonstrates that the stimulatory effect of TGF β 1 on apoptosis is also concentration dependent. In all three panels, cells were treated for 24 h prior to harvest for apoptosis ELISA. Each point represents the mean (\pm SE) of two determinations

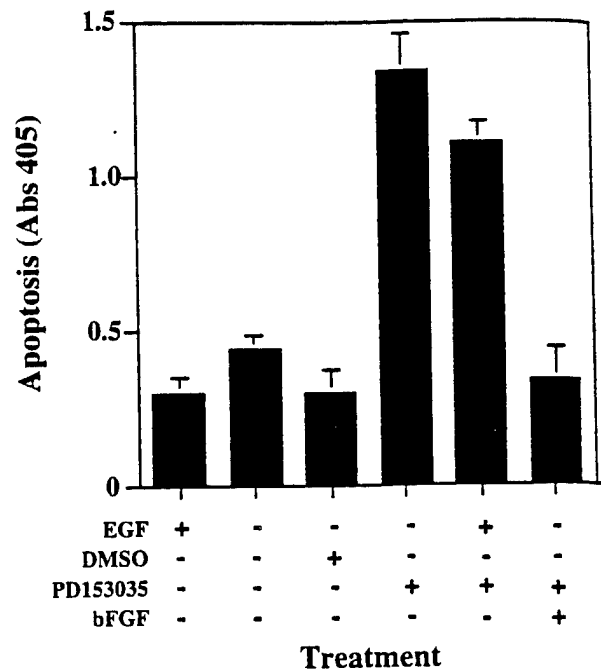


Figure 4 Induction of apoptosis in the TGF α .Myc#75 line by PD153035, a specific inhibitor of EGF receptor tyrosine kinase activity. Cells were incubated for 24 h with the indicated additions (10 ng/ml EGF or bFGF, 10 μ M PD153035, 1 μ l/ml DMSO as a control) and apoptosis was measured via ELISA assay. $n=4$ (\pm SE)

specifically downregulate the tyrosine phosphorylation status of the EGFR (Fry *et al.*, 1994). We verified this in our cell system by an anti-phosphotyrosine Western blot which showed that a 170 kb species was reduced greater than 90% in cells treated with the compound, while other phosphotyrosine bands remained constant (not shown). The TGF α /Myc#75 cells became apoptotic when exposed to PD153035 for 24 h (Figure 4). Removing EGF from the growth media of these cells did not affect viability, but exposure to PD153035 in either the presence or absence of EGF induced apoptosis. In contrast, bFGF, which acts through a different receptor tyrosine kinase, could rescue the cells from the effects of the drug.

Anchorage dependent (ADG) and anchorage independent (AIG) growth analysis of tumor derived cell lines

The TGF α /Myc#75 double transgenic cell line had the fastest ADG growth rate *in vitro* under normal growth conditions (doubling time of 16.7 h \pm 0.4 h). Myc#83 and TGF α #13 had much longer doubling times of 33.4 h (\pm 1.7) and 35.0 h (\pm 0.82) respectively when grown in normal media containing EGF (Figure 6a). Growth rates of all three cell lines were similar when EGF was replaced with TGF α (not shown). The two cell lines that overexpress TGF α (TGF α /Myc#75 and TGF α #13) were able to grow in the absence of exogenous EGF, but with a significantly reduced growth rate (Figure 5a). Two additional TGF α /Myc cell lines also exhibited relatively fast growth rates and were not dependent on exogenous EGF for growth or survival, similar to TGF α Myc#75 (not shown). In

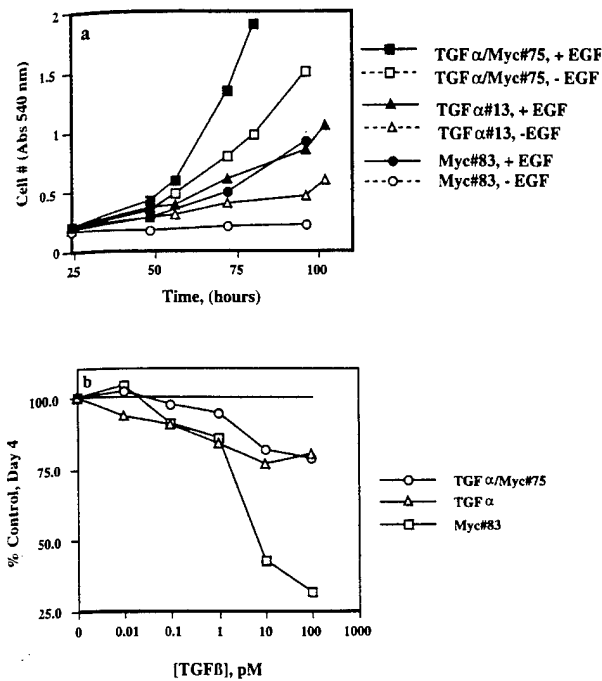


Figure 5 Co-expression of TGF α and Myc results in cooperative growth stimulus under anchorage dependent conditions. (a): Cells were grown in the presence or absence of EGF in 96 well plates for the indicated times and stained with crystal violet. (b): Cells were grown in the presence of EGF and increasing concentrations of TGF β 1 for 3 (TGF α /Myc#75) or 4 (Myc#83, TGF α #13) days and then stained with crystal violet. For (a) and (b), $n=8$ (\pm SE)

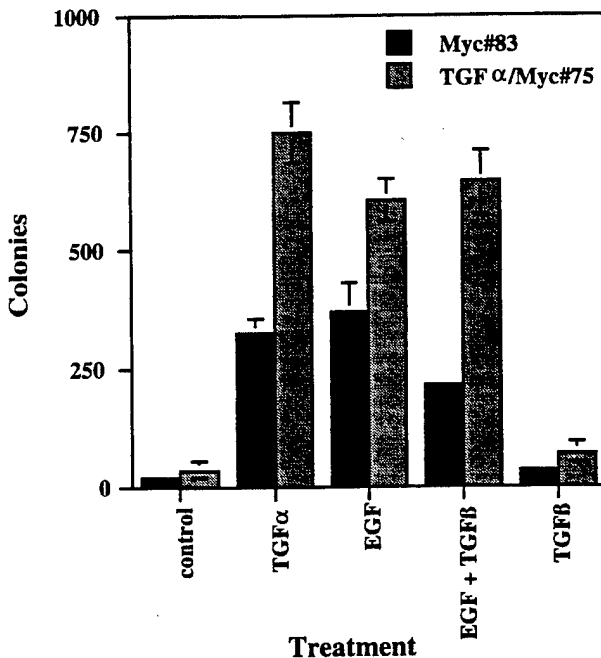


Figure 6 Co-expression of TGF α and Myc results in cooperative growth stimulus under anchorage independent conditions. Cells were suspended in 0.3% agar with 10% FCS and the following additions: TGF α (5 ng/ml), EGF (10 ng/ml), TGF β 1 (100 pM) or EGF + TGF β 1. Colonies were counted on day 7 (TGF α /Myc#75) or day 10 (Myc#83). $n=3$ (\pm SD). Results for TGF α #13 are not shown since that cell line did not grow well in soft agar under any of the above conditions

contrast, Myc#83 cells were completely dependent on exogenous EGF and showed no significant growth in its absence. However, FACS analysis demonstrated that Myc#83 cells were not arrested in G0/G1 when deprived of EGF (not shown). Those results are consistent with the hypothesis that Myc overexpressing cells are unable to withdraw from the cell cycle and undergo apoptosis in the absence of EGF.

All three cell lines exhibited concentration dependent inhibition by TGF β 1 under ADG conditions, but sensitivity to the growth factor varied (Figure 5b). At the highest concentrations (10–100 pM), TGF α /Myc#75 and TGF α #13 were marginally responsive to TGF β , with about 20% fewer cells in treated wells than in untreated controls after 3–4 days in culture. In contrast, Myc#83 cells were quite sensitive to high concentrations of TGF β 1, reflecting the observation that TGF β induces apoptosis in these cells (Figure 3a,c).

TGF α /Myc#75 and Myc#83 cells both grew well under AIG conditions in the presence of exogenous EGF or TGF α (Figure 6), whereas TGF α #13 cells grew poorly in soft agar. The effects of those growth factors on Myc#83 cells are similar to published results for other Myc-overexpressing breast cells (Telang *et al.*, 1990; Valverius *et al.*, 1990). The rate of colony formation and growth was much higher for the TGF α /Myc#75 cells, and a dose response curve showed that those cells were extremely sensitive to TGF α , with optimal induction by only 0.1 ng/ml of the growth factor. Maximal colony formation by the Myc#83 line occurred with 10–30 ng/ml TGF α (not shown). Addition of TGF β 1 significantly reduced the number of Myc#83 colonies stimulated by addition of EGF, but had no effect on TGF α /Myc#75 colony formation (Figure 6).

Tumorigenicity of tumor derived cell lines

Cells were injected into female *nu/nu* mice to establish their *in vivo* tumorigenicity and growth rate. All three cell lines grew readily in nude mice, but with different latency times. The double transgenic line TGF α /Myc#75 formed tumors with a latency period of only 4 weeks, while the single transgenic TGF α #13 and Myc#83 lines formed tumors with a latency period of about 9 weeks. None of the cell lines appeared to have metastatic capabilities over the period of time the tumors were allowed to grow (About 2 months for TGF α /Myc#75 and 3 months for TGF α #13 and Myc#83).

Discussion

Myc overexpression (achieved by gene amplification, translocations and other means) has been strongly implicated in the genesis of many types of human tumors including breast cancer (Bonilla *et al.*, 1988; Cole, 1986; Escot *et al.*, 1986; Garcia *et al.*, 1989; Mariani-Constantini *et al.*, 1988). However, since deregulated c-Myc expression can promote cell death *via* apoptosis, it is likely that the apoptotic pathway(s) induced by c-Myc must be inhibited or inactivated to achieve aggressive tumor formation. That may be accomplished, directly or indirectly, by secondary

events which alter the cell environment (such as growth factor secretion) or gene expression (such as mutations in downstream effectors). In accordance with that hypothesis, we detected apoptosis only in mammary gland tumors that expressed the c-myc transgene alone without the TGF α transgene. Tumors from TGF α single transgenic or TGF α /c-myc double transgenic mice did not contain apoptotic cells. Van Dyke and co-workers have proposed a similar 'multi-hit' hypothesis of tumor formation based on studies of SV40 T antigen-induced brain tumors (Symonds et al., 1994). In that system they found that wild type T antigen induced rapidly growing, aggressive tumors, whereas a mutated form of the protein which only interfered with pRb function produced very slow growing tumors which displayed a high percentage of apoptotic cells. In contrast, expression of the mutant T antigen in a p53-null background resulted in tumors which were indistinguishable from those induced by the wild type protein. Taken together, the results suggest that the first event in cancer initiation stimulates both proliferation and apoptosis and that a secondary event which blocks apoptosis is necessary for aggressive tumor formation.

Cell lines derived from the tumors provided an *in vitro* confirmation of our *in vivo* observations. Apoptosis was observed only in the cell line derived from a Myc single transgenic animal (Myc#83), while the two cell lines overexpressing TGF α (TGF α /Myc#75 and TGF α /Myc#13) did not undergo apoptosis under normal culture conditions. TGF α /Myc#75 cells only became apoptotic when exposed to a specific inhibitor of EGF receptor tyrosine kinase activity (PD153035), suggesting that these cells were dependent on autocrine stimulation by TGF α for survival. Analogously, exogenous TGF α inhibited apoptosis in the Myc#83 cells. Results from both mammary gland tumors and their derived cell lines are therefore in good agreement and mirror previous studies which have shown that apoptosis was induced when c-Myc was overexpressed (Askew et al., 1991; Evan et al., 1992).

Our findings could provide at least a partial explanation for why TGF α and c-Myc cooperate in mammary gland tumorigenesis in the powerful way we described previously (Amundadottir et al., 1995). In that study, single transgenic virgin c-Myc mice developed mammary gland tumors at around 10 months of age and virgin single transgenic TGF α animals never developed mammary gland tumors. In contrast, double transgenic TGF α /c-myc mice exhibited a tumor latency that was shortened to only 66 days, and mammary gland tissue from mice as young as 3 weeks grew readily as a tumor in nude mice. A similar synergism was observed in a WAP-TGF α \times WAP-Myc double transgenic model (Sandgren et al., 1995). In that report, as well as a study involving the MT100TGF α strain used in our model (Smith et al., 1995), it was also observed that TGF α overexpression inhibited post-lactational involution, a process dependent on apoptosis. Those observations lend further credence to the hypothesis that TGF α can act as a survival factor in the mammary gland.

TGF α has not been shown previously to inhibit c-Myc-mediated apoptosis but insulin like growth factors (IGF-I and IGF-II) and platelet derived growth factor (PDGF) acted as survival factors for Rat-1 fibroblasts which overexpressed c-Myc (Harrington et al., 1994).

Interestingly, EGF could not inhibit c-Myc-induced apoptosis in those cells, even though they expressed functional EGF receptor. Although the reasons for this discrepancy are not known, it is most likely the result of cell type specificity. That assumption is supported by the observation that EGF could act as a survival factor for nontransformed mammary epithelial cells which were serum starved or grown to confluency (Merlo et al., 1995). Our data from the Myc#83 tumor cell line indicate that EGF, bFGF and IGF-I can also inhibit c-Myc-mediated apoptosis, suggesting that they could potentially cooperate with c-Myc in mammary tumorigenesis as well.

Mutations in the p53 gene may also cooperate with c-Myc in tumorigenesis, since p53 has been shown to be required for Myc-mediated apoptosis in some, but not all cases (Sakamuro et al., 1995; Hsu et al., 1995; Hermeking and Eick, 1994). In addition, upregulation of bcl-2 gene expression (a death suppressor), downregulation of bax gene expression (a death promotor) or abrogated expression of other proteins involved in Myc-induced apoptosis might be involved. Bcl-2 has been shown to cooperate with c-Myc in transformation *in vitro* and *in vivo* (Bissonette et al., 1992; Fanidi et al., 1992; Strasser et al., 1990; Wagner et al., 1993), and Bax gene expression may be regulated by c-Myc, since its promotor contains several putative Myc binding sites (Miyashita and Reed, 1995). However it is not known whether TGF α can directly influence the apoptotic machinery of the cells. The effects of TGF α and TGF β 1 on expression of p53, Bcl-2, Bax and other proteins involved in apoptosis are currently being investigated.

A cooperative growth stimulus also appears to contribute to the synergism between TGF α and c-Myc in mammary tumorigenesis. The doubling time of TGF α /Myc#75 cells was approximately half that of cells expressing only one of the transgenes, and their growth in soft agar and nude mice was much more aggressive than the other two cell lines. Taken together, the data suggest that one aspect of the positive interaction between TGF α and c-Myc in tumorigenesis might be via upregulation of genes that control progression through the cell cycle. Collectively, these gene products might account for high growth rates and malignant progression. Potentially, coexpression of TGF α and c-Myc could also alleviate negative control on growth and transformation.

In vitro studies with the tumor-derived cell lines suggest that may be the case. TGF β 1 inhibits the growth of most epithelial cells, including mammary epithelial cells (Daniel et al., 1989; Jhappan et al., 1993; Pierce et al., 1993; Silberstein and Daniel, 1987; Valverius et al., 1989; Zugmaier et al., 1989). However, the TGF α /Myc#75 line was only marginally responsive to TGF β 1 in ADG assays and was insensitive to the growth factor under anchorage independent conditions. In contrast, Myc#83 cells grown on plastic were quite sensitive to TGF β , and their rate of colony formation in soft agar was significantly reduced in the presence of TGF β . Apoptosis assays revealed that Myc#83 cells were not merely growth-inhibited by TGF β , but rather they were stimulated to undergo apoptosis, even in the presence of a survival factor (EGF). Induction of apoptosis by TGF β has previously been reported for



some other cell types, including normal and malignant ovarian epithelial cells (Havrilesky *et al.*, 1995), endometrial cells (Rotello *et al.*, 1991), rat prostate cells (Martikainen *et al.*, 1990), normal and transformed hepatocytes (Oberhammer *et al.*, 1992), and leukemia cells (Selvakumaran *et al.*, 1994a,b; Taetle *et al.*, 1993). Furthermore, mammary glands from pregnant WAP-TGF β transgenic mice showed high levels of apoptosis with a subsequent lack of secretory lobule development (Korden *et al.*, 1995). Since TGF β expression is elevated in human tumor cells compared to normal mammary tissue and protein levels are positively correlated with disease progression (Gorsch *et al.*, 1992), breast tumor cells must develop the ability to grow in the presence of relatively high concentrations of TGF β . The results from our *in vitro* studies indicate that cells which overexpress only c-Myc would not have that ability.

Our results suggest a new role for TGF α as a survival factor in breast cancer. We therefore conclude that the strong synergism of TGF α and Myc in mammary gland tumorigenesis is in fact due not only to a dual growth stimulus, but to the ability of TGF α to suppress a negative aspect of Myc overexpression.

Materials and methods

Transgenic animals

Transgenic mice were generated as described previously by mating the MT100 TGF α strain to the MMTV-c-myc M strain (Amundadottir *et al.*, 1995). The four resulting genotypes were: TGF α /c-myc double transgenic mice, TGF α single transgenic mice, c-myc single transgenic mice, and wild type mice. Tumors were observed to form in each strain as follows: in TGF α /c-myc virgin females and males with a latency of about 66 days; in multiparous single transgenic TGF α females with a latency of about 10 months; and in virgin or multiparous single transgenic c-myc females with a latency of about 10 months.

Detection of apoptosis in tumors

The occurrence of apoptosis in mammary gland tumors was detected by *in situ* nick end-labeling of nucleosomal DNA fragments (Ansari *et al.*, 1993). Paraffin embedded tumor sections were deparaffinized in a series of xylene and ethanol washes. This was followed by a 0.3% H₂O₂ treatment for 30 min to inactivate endogenous peroxidases, after which slides were immersed in buffer A for 5 min (50 mM Tris pH 7.5, 5 mM MgCl₂, 0.76 mM 2-mercaptoethanol and 0.005% BSA). Subsequently, slides were incubated with Klenow enzyme (50 U/ml, Boehringer Mannheim, Indianapolis, IN), 5 μ M biotinylated dUTP (Boehringer Mannheim) and 2 μ M dATP, dGTP and dCTP (Promega, Madison, WI) in buffer A for 60 min at 37°C. After washing slides in PBS, they were incubated with solution AB (ABC kit, Biomed, Foster City, CA), rewashed in PBS and stained with diaminobenzidine (DAB, Sigma, St. Louis, MO). Finally, the slides were counterstained with aqueous methyl green (Sigma), dehydrated and mounted.

Primary cultures from tumors

Tumor bearing transgenic animals were sacrificed and tumors were excised aseptically. Tumors were then cut into about 1 mm³ pieces and digested overnight at 37°C in

DMEM-F12 (Biofluids, Rockville, MD) with 10% fetal calf serum (FCS, Biofluids), 5 ng/ml EGF (Upstate Biotechnology Incorporated [UBI], Lake Placid NY), 10 μ g/ml insulin (Biofluids) supplemented with 1 mg/ml collagenase type 1A (Sigma), antibiotics and fungizone (Biofluids). The following day cells were pelleted by centrifugation and washed three times in growth media (DMEM-F12 with 2.5% FCS, 5 ng/ml EGF, 10 μ g/ml insulin and antibiotics). Cells were plated at 1–2 \times 10⁶ cells per T75 flask in growth medium. Fungizone was used in the cell medium for the first 2–3 weeks to prevent fungal contamination. Media were changed every 2–3 days and fibroblast overgrowth was prevented by differential trypsinization of cultures until fibroblasts were no longer observed (based on morphology). When epithelial cells were about 60–70% confluent (after 2–3 months of growth), the cultures were passed at 1:2 dilutions with dispase (Boehringer Mannheim). At later passages cells were split with trypsin (Gibco BRL, Gaithersburg, MD) twice a week at 1:5 to 1:50, depending on the line.

RNA isolation and analysis

Cultured cells were harvested by rocking plates with guanidine thiocyanate for 5–10 min. RNA was extracted with acid phenol and precipitated with isopropanol. Ten μ g total RNA were electrophoresed through 1.2% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Amersham, Arlington Heights, IL) and probed with ³²P-labeled riboprobes generated with the Riboprobe II Core System (Promega) from the following plasmids: pTGF α -pGEM3Z linearized with HindIII and transcribed with T7; c-myc-pGEM4Z linearized with EcoRI and transcribed with T7; and pmK14-pGEM3 (mouse keratin#14) linearized with HindIII and transcribed with SP6 polymerase. Labeled pBluescript poly-linker was hybridized with the 28S RNA as an internal loading control for Northern analysis (Witkiewicz *et al.*, 1993).

Growth assays

Anchorage dependent growth assays were performed in 96-well plates (Costar, Cambridge, MA). Cells were plated at a density of 1500 cells per well and were cultured in normal growth media, with or without EGF (10 ng/ml). At various time points (two per day for 4 days), the plates were stained with crystal violet (Sigma, 0.5% in 30% MeOH), rinsed with water and dried. At the end of the experiment, the dye was redissolved in 0.1 M sodium citrate in 50% ETOH and the absorbance at 540 nm was measured with an MR700 plate reader (Dynatech Laboratories Inc). Doubling times were calculated from the slope of the line generated by plotting log (absorbance) vs time. In order to test the sensitivity of the cells to TGF β 1, cells were also plated in normal growth media with EGF plus TGF β 1 (0.01–100 pM [R&D Systems, Minneapolis, MN]). When TGF β was used as a treatment, the media were changed every other day and cell number was measured on day 3 (TGF α /Myc#75) or 4 (TGF α #13 and Myc#83).

The cell lines were also tested for their ability to grow under anchorage independent conditions. Cells (10⁴) were suspended in 0.3% Bactoagar (Difco, Detroit MI) and seeded into 35 mm dishes over a 0.8% agar base layer in IMEM plus 10% FCS with the following additions: TGF α (10 ng/ml, UBI), EGF (10 ng/ml), TGF β 1 (100 pM), or TGF β 1 and EGF together. Every other day, 300 μ l of media with growth factors was added to each plate. After 7–10 days, colonies larger than 40 μ m in diameter were counted with an Omnicon 3600 image analysis system (Artek Systems Corp., Farmingdale NY).

Tumorigenicity of cell lines

Cell lines were injected into female NCR *nu/nu* mice in order to determine whether they retained tumorigenic potential. About 10^6 cells were injected subcutaneously (between nipples number 2 and 3, and 4 and 5) into nude mice under anesthesia. Two to four sites were injected per animal.

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Acknowledgements

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